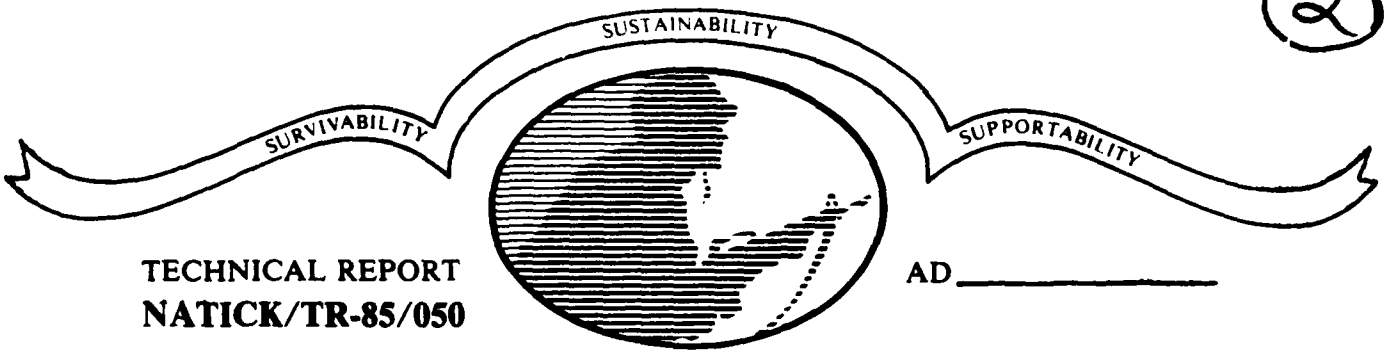

TECHNICAL REPORT
NATICK/TR-85/050

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BIOTRANSFORMATION OF WASTE WATER CONSTITUENTS FROM BALL POWDER PRODUCTION

BY
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AND
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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) This report describes the biotransformation of compounds present in various waste streams from the manufacture of ball powder. In the presence of rich nutrients the compounds diethylphthalate, dibutylphthalate, 2-ethylhexanoate, diphenylamine, 2-nitrodiphenylamine, diethyldiphenylurea (ethylcentralite), and 2,4-dinitrotoluene were biotransformed by a consortium of microorganisms from an inoculum of anaerobic and/or activated sewage sludge. All compounds were susceptible under one set of conditions or another (i.e., aerobic or anaerobic, static culture or continuous culture). Some intermediates were identified.

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PREFACE

This report describes studies on the biotransformation of a number of compounds present in waste waters from the manufacture of ball-grain powder. The studies were conducted in static and continuous culture modes and both aerobic and anaerobic conditions.

An attractive and economical method of detoxifying of wastes is to expose the materials to a microbiological process as part of the total treatment. The hope is that the pollutants will be completely biodegraded into innocuous products or that they will be biotransformed into nontoxic compound. The data obtained in this study are needed for the development of a treatment facility and to ensure that the waste waters receive an adequate treatment to meet environmental standards.

This work was supported by the US Army Toxic and Hazardous Materials Agency under project number 1L162720D048, Task W-72.

We thank Carmine DiPietro for his GC/MS analyses and Barry Greene and Kathy Cofelice for their technical assistance.

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INTRODUCTION

During the production of ball-grain powder, various by-products are produced and become discharged into the environment. In order to develop facilities to treat adequately the waste effluents resulting from the manufacture of ball powder, the susceptibility to biodegradation of compounds present in these effluents must be established. Microbial treatment has been proposed as a possible method to reduce or eliminate these environmental pollutants. Many of the compounds identified in waste streams from the various production lines involved in ball powder manufacture are known to be readily biodegradable and thus present no hazard. Other compounds, the biotransformability of which little or nothing is known, may be susceptible to biodegradation and thereby rendered nontoxic. This study deals specifically with the compounds listed in Table 1.

TABLE 1. Compounds Present in Ball Powder Manufacturing Waste Waters Investigated in this Study

Diethylphthalate
Dibutylphthalate
2-Ethylhexanoate
Diphenylamine
2-Nitrodiphenylamine
Ethylcentralite
2,4-Dinitrotoluene
Nitrocellulose

A number of investigators[1,2,3,4,5] have reported the biodegradation of phthalates by mixed cultures from activated sludges and from marine sediments, and by pure culture isolates identified as species of genera including: Acinetobacter, Pseudomonas, Bacillus, Nocardia and Arthrobacter. The anaerobic biotransformation of phthalates has been reported to occur under denitrification conditions[1,2]. Diethyl- and dibutylphthalate were included in this study to confirm their biodegradation in anaerobic systems under denitrification conditions.

The biodegradation of compounds such as the N-phenylbenzenamines (diphenylamine, 2-nitrodiphenylamine) has not been studied in great detail.

Gardner et al.[6] reported on studies using ^{14}C-labeled diphenylamine in a mixed microbial consortium from sewage sludge. Several intermediates were detected, one of which was identified as 4-hydroxydiphenylamine.

Previous work has shown that in a microbial system, the nitro groups of 2,4-DNT are readily reduced to amino groups[7,8] which may become acetylated or partially reduced. Nitro groups (nitroso, hydroxylamino) may undergo dimerization and subsequent formation of azoxy compounds.[7] It has been reported that a Pseudomonas species isolated from 2,4-DNT wastewaters could grow in a basal salts medium containing 2,4-DNT as the sole carbon source.[9]

Studies on the fate of nitrocellulose particulates present in the wastewaters from ball powder manufacture have been reported.[10] No information was available on biodegradation studies with 2-ethylhexoate or ethylcentralite.

Since nitrate is present in most munitions waste waters and is known to cause environmental problems, denitrification studies were conducted concomitantly with biotransformation studies on the organic compounds.

MATERIALS AND METHODS

Cultures and Media. Biodegradation studies were carried out either in nutrient broth (Difco) or in a basal salts medium consisting of: K_2HPO_4, 0.87 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; NaCl, 0.05 g; $CaCl_2$, 0.01 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; $CuSO_4 \cdot 7H_2O$, 0.01 g; $MnSO_4 \cdot H_2O$, 0.01 g; and Na_2MoO_4, 0.002 g. The carbon source was methanol. Potassium nitrate was added to some cultures to bring the final nitrate concentration to 500 ppm or to 2480 ppm (0.04 M).

Batch Cultures. For anaerobic studies, the culture flasks were filled to approximately 95% of their capacity and incubated as static cultures at 37°C. The inoculum was prepared by diluting anaerobic sewage sludge (obtained from the Nut Island Sewage Treatment Plant, Boston, MA) with an equal volume of water and filtering through glass wool. A 1% inoculum was used.

For aerobic studies, the flasks were filled to 10% capacity and incubated in a shaking water bath (Aquatherm Model G-86, New Brunswick Scientific Co., Inc., Edison, NJ) at 37°C. Aerobic inocula were prepared by diluting anaerobic sewage sludge with an equal volume of activated sludge (Marlborough Easterly Sewage Treatment Plant, Marlborough, MA) and filtering through glass wool. A 1% inoculum was used.

Due to the relative insolubilities of the compounds studied, the proper amounts were weighed, added to a flask previously sterilized by autoclaving, and dissolved in a small amount of acetone with warming. The acetone was removed by evaporation under a stream of nitrogen leaving a thin film of material coating the surface of the flask. Medium just removed from the autoclave was added aseptically to the flask containing the film of compound, the contents were heated and stirred vigorously until solution was attained. The medium was allowed to cool to <40°C before inoculation. Samples were removed at various times, centrifuged, filtered through 0.45 u membrane filters, and analyzed by High Performance Liquid Chromatography (HPLC).

Continuous Cultures. Continuous culture systems were set up using a two-liter Erlenmeyer flask as the influent reservoir and a one-liter filter flask as the reaction vessel. A peristaltic pump (Rainin Instrument Co., Inc., Woburn, MA) was used to pump the medium through the tubing and the medium break-tubes to the culture vessel. For aerobic systems the culture flask was aerated by a Whisper 700A aquarium pump (Willinger Bros., Inc., Ft. Lee, NJ). Samples of effluents were removed three times weekly, centrifuged, filtered through Nylon 66 membrane filters (Rainin Instrument Co., Inc., Woburn, MA), and the filtrates analyzed by HPLC. Some samples were absorbed by passage through membrane filters and were analyzed by HPLC without filtering.

When it was desirable to concentrate the biotransformation products the culture medium was clarified to remove cells by centrifugation and then continuously extracted for 24 hours with methylene chloride. The methylene chloride was removed from the extract and this residue was taken up in several mL of methanol or acetonitrile.

HPLC Analysis. HPLC analyses were conducted using a Waters liquid chromatograph system equipped with two Model 6000A pumps, a Model 710 automated sample processor, a Model 730 data module, a Model 721 system controller, a Z-Mod radial compression separation system, and a Model 440 wavelength detector (Millipore Waters Chromatography Division, Milford, MA). Analyses were carried out using either a 3.9 mm x 30 cm uBondapak C_{18} stainless steel column (analytical) or a 8 mm x 10 cm Radial Pak C_{18} cartridge (semi-prep). Solvents used were high purity methanol and acetonitrile (Burdick and Jackson Labs., Inc., Muskegon, MI) and high purity water from a Milli-Q system (Millipore Corp., Bedford, MA). Table 2 lists the parameters used for detection, separation and isolation of the compounds described in this report.

Nitrate Analysis. Nitrate determinations were performed with a Model 901 Ionalyzer using a Model 93-07 nitrate electrode (Orion Research, Inc., Cambridge, MA). High concentrations of organic matter affected the reliability of the readings. An alternate method used the HPLC equipped with an 8 mm x 10 cm Radial-Pak strong anion exchange (SAX) column in the Z-Mod. The solvent was 2.5 mM phosphate buffer, pH 7.0, at a flow rate of 3 mL/min, and the detector was set at 229 nm.

Oxidation-Reduction Potential (E_h) Measurements. E_h measurements were obtained using a Model 901 Ionalyzer and a Model 96-78 platinum redox electrode (Orion Research, Inc., Cambridge, MA).

Total Organic Carbon (TOC). TOC measurements were conducted on a Beckman Model 915B Tocamaster (Beckman Instruments, Inc., Carlsbad, CA). Samples were clarified by centrifugation, filtered through membrane filters, and acidified to pH 2 to liberate CO_2. Organic carbon was determined by injection of 20 uL of the acidified sample, conversion to CO_2 at 950°C, and detection with an IR detector.

TABLE 2. Summary of HPLC parameters used for separation of various compounds.

Compound	Retention Time (min) AN[a]	Retention Time (min) SP[b]	Column	Wave Length (nm)	Flow Rate (mL/min)	Solvent
dibutylphthalate	6.85	-	AN-R[c]	229	2.5	MeOH:H_2O (80:20)
diethylphthalate	6.46	-	AN-R	229	2.5	MeOH:H_2O (60:40)
diphenylamine	6.50	49.31	AN-S[d]	280	2.0	MeOH:H_2O (65:35)
4-hydroxydiphenylamine	-	35.98	or			
N,N'-diacetyl-o-phenylene-diamine	-	15.18	SP	254	1→3→1	
phenazine	-	44.00				MeOH:H_2O gradient from 50:50 to 65:35
2-nitrodiphenylamine	6.16	60.31	AN-R	254	2.5	MeOH:H_2O (80:20)
2-aminodiphenylamine	-	40.73	or			
N-phenylbenzimidazole	-	48.31	SP	254	1→3→1	MeOH:H_2O gradient from 50:50 to 65:35
2-ethylhexanoate	8.35	-	AN-R	229	3.0	AcCN:H_2O gradient from 65:35 to 100:0
centralite	7.11	40.66	AN-R	254	2.0	MeOH:H_2O (75:25)
N-ethylaniline	3.70	18.70	or			
ethyldiphenylurea	4.60	28.10	SP	254	2.5	MeOH:H_2O gradient from 55:45 to 70:30
2,4-dinitrotoluene	10.10	-	AN-S	230	2.0	MeOH:H_2O (40:60)
2-acetamido-4-nitrotoluene	3.86	-	or			
4-acetamido-2-nitrotoluene	7.25	-	SP	230	2.5	MeOH:H_2O (40:60)
2-amino-4-nitrotoluene	5.75	-				
4-amino-2-nitrotoluene	5.21	-				
2,4-dinitrotoluene	3.03	-				

[a]AN = analytical columns
[b]SP = semi-preparative uBondapak C_{18} column, 7.8 mm x 30 cm steel column
[c]AN-R = analytical uBondapak C_{18} column, 8 mm x 10 cm Radial-Pak cartridge
[d]AN-S = analytical uBondapak C_{18} column, 3.9 mm x 30 cm steel column

Preparation of N-Phenylbenzimidazole. The compound was synthesized from N-phenyl-o-phenylenediamine and formic acid according to the procedure described by Taylor.[11]

Ethylhexanoic Acid. Analysis of 2-ethylhexanoic acid involved preparing the phenacyl ester and using HPLC to determine disappearance. Samples were treated with Phenacyl-8 (Pierce Chemical Co., Rockford, IL) according to the following procedure. A sample (5 mL) from the culture flask was centrifuged to remove cells. A 1.0 mL sample of this was acidified with 1 to 2 drops of 1 N HCl. Methylene chloride (5 mL) was added, the container capped, and the contents shaken vigorously for 3 minutes. The bottom layer was removed, several drops of water were added, the mixture was made basic with 1 N KOH in methanol, the solution was evaporated to dryness under N_2, 0.1 mL of the Phenacyl-8 reagent and 2.9 mL of acetonitrile were added to the dry residue, the tubes were capped and heated in a heating block at 80°C for 20 min with intermittent shaking, and the solution was injected directly into the HPLC for analysis.

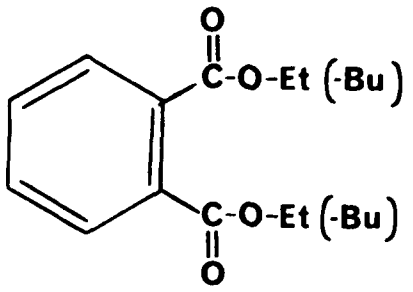
Radioactive 2,4-DNT Experiment. To a continuous culture system, fed full-strength nutrient broth containing 10 mg/L of 2,4-DNT, was added 10 uCi of uniformly labeled [^{14}C]2,4-DNT, 18 mCi/mmole, obtained from California Bionuclear Corp., Sun Valley, CA. Both aerobic and anaerobic systems were set up as already described except that the vapor phases of the effluent streams were exhausted through several traps, consisting of 0.1 N HCl, 0.1 N NaOH, and water.

RESULTS

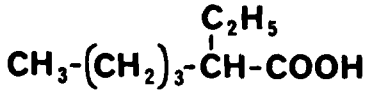
Each compound listed in Table 1 was investigated as to its susceptibility to microbial degradation and/or biotransformation under both aerobic and anaerobic conditions in both static (batch) and continuous culture systems. Figure 1 shows the structures of three of these compounds, all of which disappear rapidly when incubated under aerobic conditions.

Phthalate Esters. Figure 2 shows the disappearance of 10 mg/L (10 ppm) of dibutylphthalate incubated under aerobic conditions in a medium consisting of basal salts made up in lake water and containing 1.4 mL of methanol per liter. The concentration dropped from 10 ppm to below 1 ppm within 10 days. After 10 days no dibutylphthalate could be detected. No intermediates or metabolites were detected at any stage during the disappearance.

A different picture presented itself when the system was incubated under anaerobic conditions (Fig. 3). Over an extended period of time (90 days) dibutylphthalate disappeared from the culture flasks containing the same basal salts medium as for the aerobic system. Midway through the experiment (day 50) the system displayed a redox potential (E_h) of -177 millivolts (mV) but by the end of the experiment the E_h had risen to -26 mV, which, though still considered anaerobic, was high enough to question whether in the latter stages of the experiment, the decrease in dibutylphthalate was, in fact, due to the presence of organisms metabolizing aerobically.



diethylphthalate (dibutyl)



2-ethylhexanoic acid

Figure 1. Chemical formulae of diethyl- and dibutylphthalate and for 2-ethylhexanoic acid.

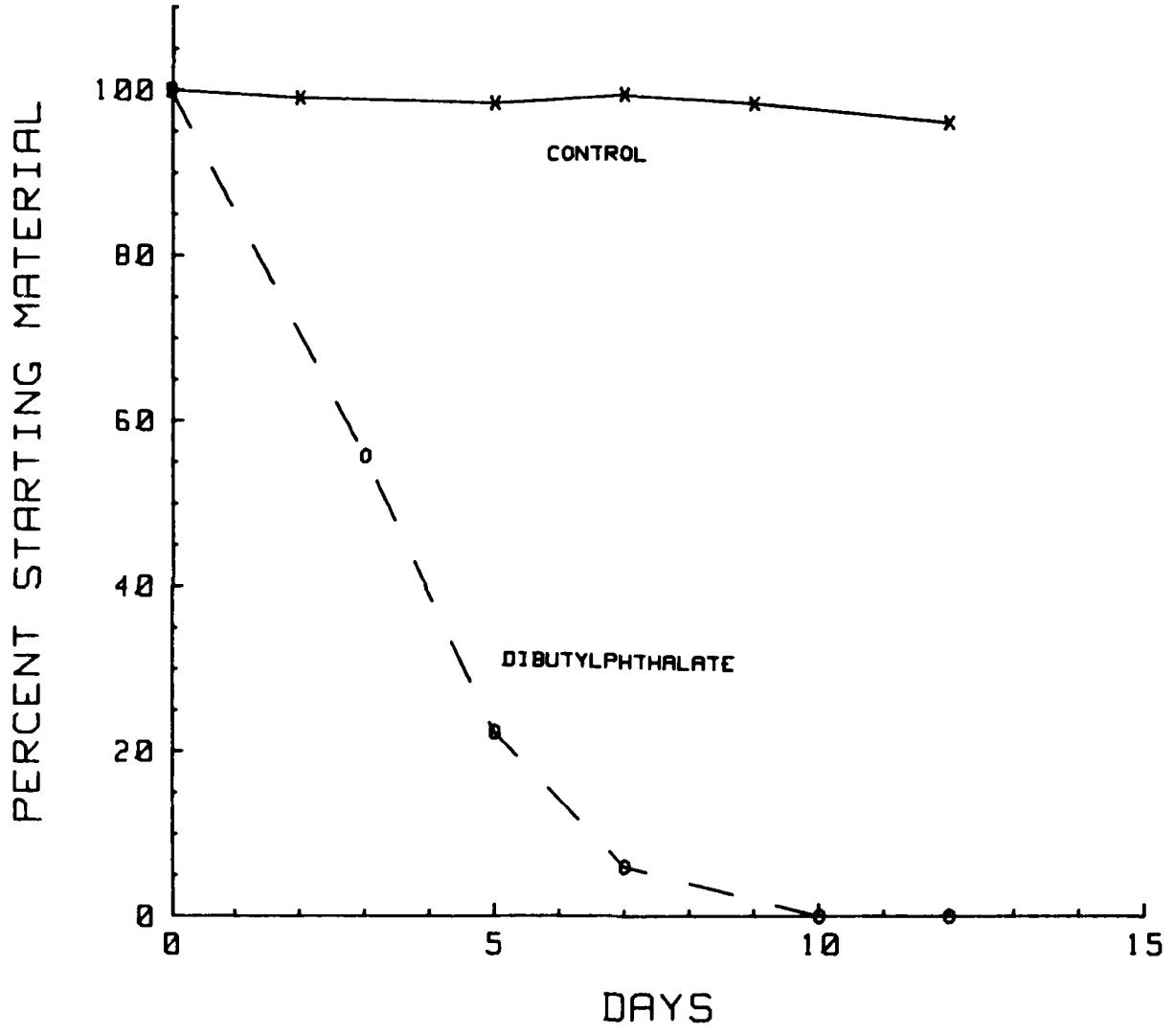


Figure 2. Disappearance of dibutylphthalate in a static culture under aerobic conditions.

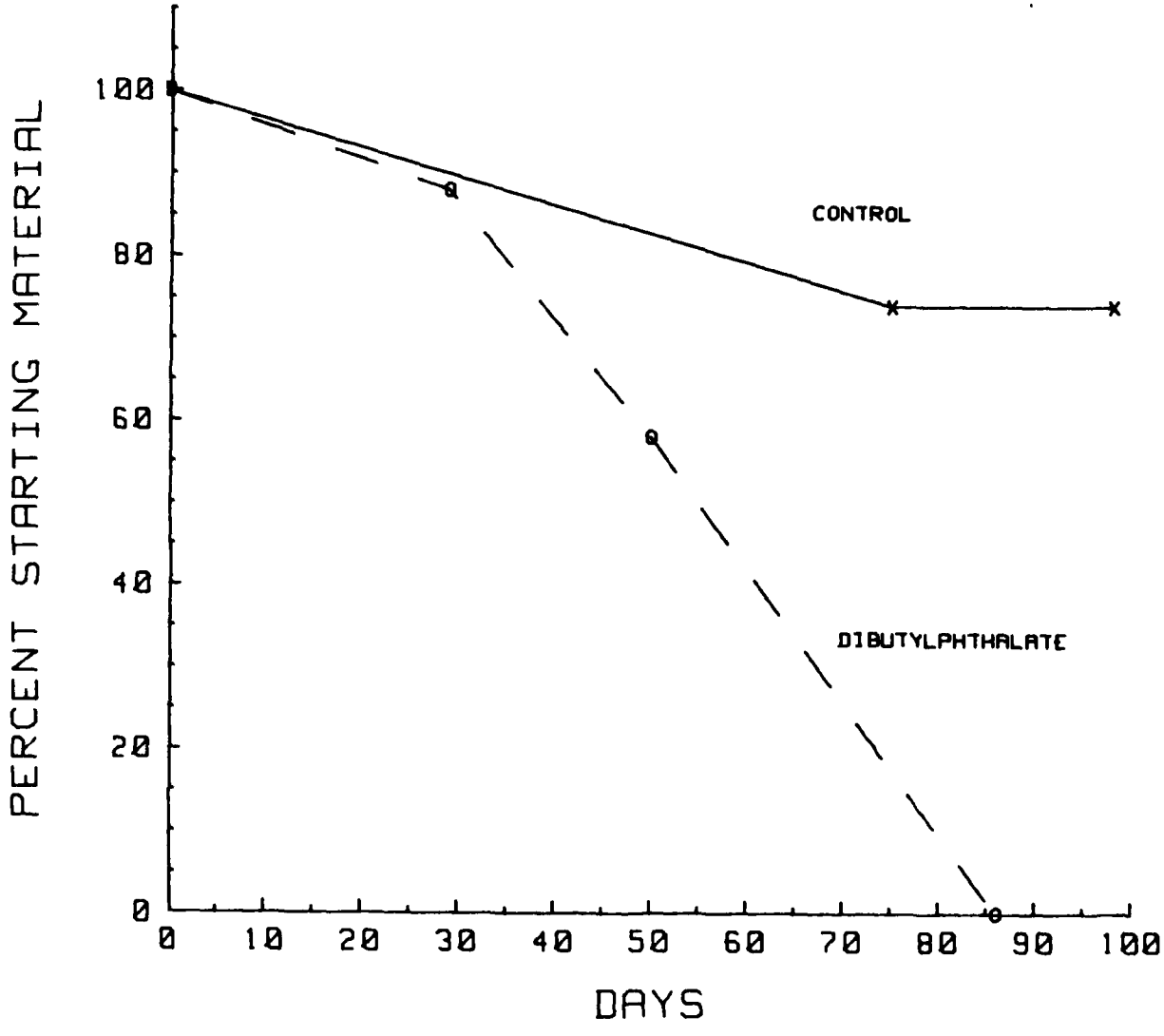


Figure 3. Disappearance of dibutylphthalate in a static culture under anaerobic conditions.

The disappearance of diethylphthalate in a basal salts-methanol medium under aerobic conditions was slower than for dibutylphthalate (Fig. 4). Disappearance under anaerobic conditions was about the same as for dibutylphthalate (Fig. 5). With diethylphthalate, the E_h at the conclusion of the experiment was -117 mV. No intermediates were detected from either ester whether under aerobic or anaerobic conditions.

The results of a long-term continuous culture experiment with diethylphthalate are shown in Fig. 6. The medium used in this experiment was 10% of full-strength nutrient broth (or 0.8 g/L), which yields a total organic carbon (TOC) concentration of 500 ppm. An average retention time of 4 days was used. No diethylphthalate was detected in effluent samples throughout the course of the aerobic continuous culture experiment (i.e., there was 100% disappearance). At day 31 (arrow a) the concentration of nutrient broth was lowered to 0.4 g/L; at day 59 (arrow b) the medium was changed to a basal salts-methanol medium in lake water; at day 85 (arrow c) the methanol was omitted and distilled water was substituted for lake water; at day 98 (arrow d) the salts were omitted: and at day 150 (arrow e) ultrapure Milli-Q water was the only other ingredient in the influent solution besides the diethylphthalate itself. Even after 170 days no diethylphthalate could be detected in the effluent.

Under anaerobic conditions the diethylphthalate did not break down as well as under aerobic conditions (Fig. 6). As the ester fed into the system the concentration built up (i.e., decreased in percent loss) and then degradation began to occur. Some problems were encountered with the phthalates being absorbed by the silicon tubing used to pump the influent. We found that between 75% and 90% of the material was passing through. An average retention time of 6 days was used in this system, due to the poorer breakdown. At 60 days (arrow f) the concentration of nutrient broth was increased five-fold from 0.8 g/L to 4 g/L (2500 ppm TOC), and the retention time was reduced to five days. There appeared to be a slight increase in disappearance between 70 and 80 days. At day 121 the retention time was increased to seven days and there was a sudden increase in disappearance. No intermediates were detected in the effluent during the course of the experiment. The system maintained an E_h of -250 to -300 mV throughout and carried out >99% denitrification of 500 ppm of nitrate.

2-Ethylhexanoate. This compound disappeared rapidly from aerobic static cultures within three days (Fig. 7); whereas, no disappearance was noted under anaerobic conditions. The medium used was 0.8 g/L of nutrient broth. Typically, the more carbon source present the faster the compound disappeared. No intermediates were detected. In continuous cultures disappearance was rapid under aerobic conditions but slow if at all under anaerobic conditions.

Diphenylamine (N-Phenylbenzenamine)(DPA). The structures of DPA and several identified metabolic products are shown in Fig. 8. The results of an experiment carried out under anaerobic conditions in which the nutrient source was full-strength nutrient broth (8 g/L), revealed little or no breakdown occurred under these conditions (Fig. 9). Under aerobic conditions there was a rapid disappearance of DPA (Fig. 10) and a corresponding increase in the appearance of several intermediates (a & b). The HPLC separation of these

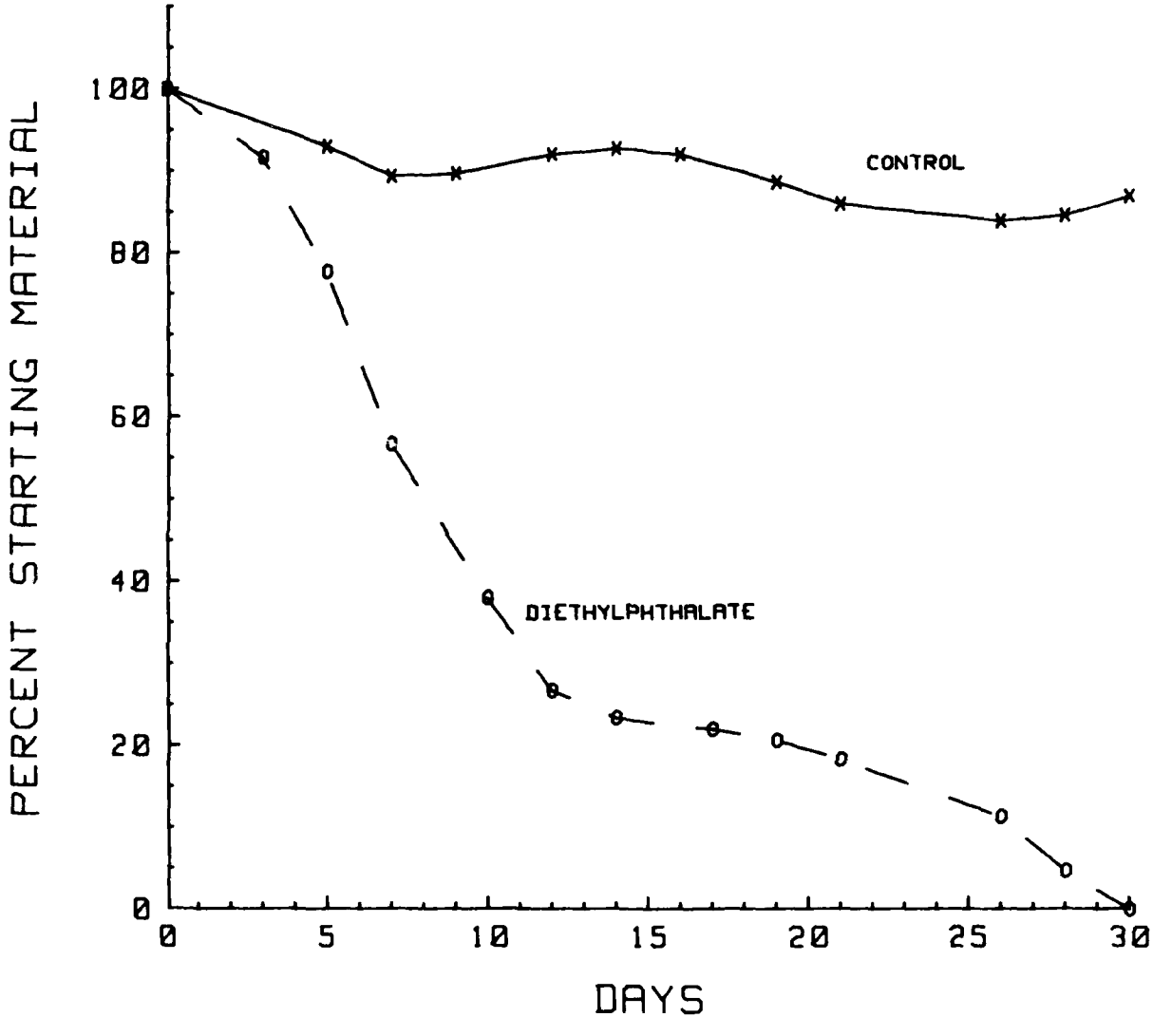


Figure 4. Disappearance of diethylphthalate in a static culture under anaerobic conditions.

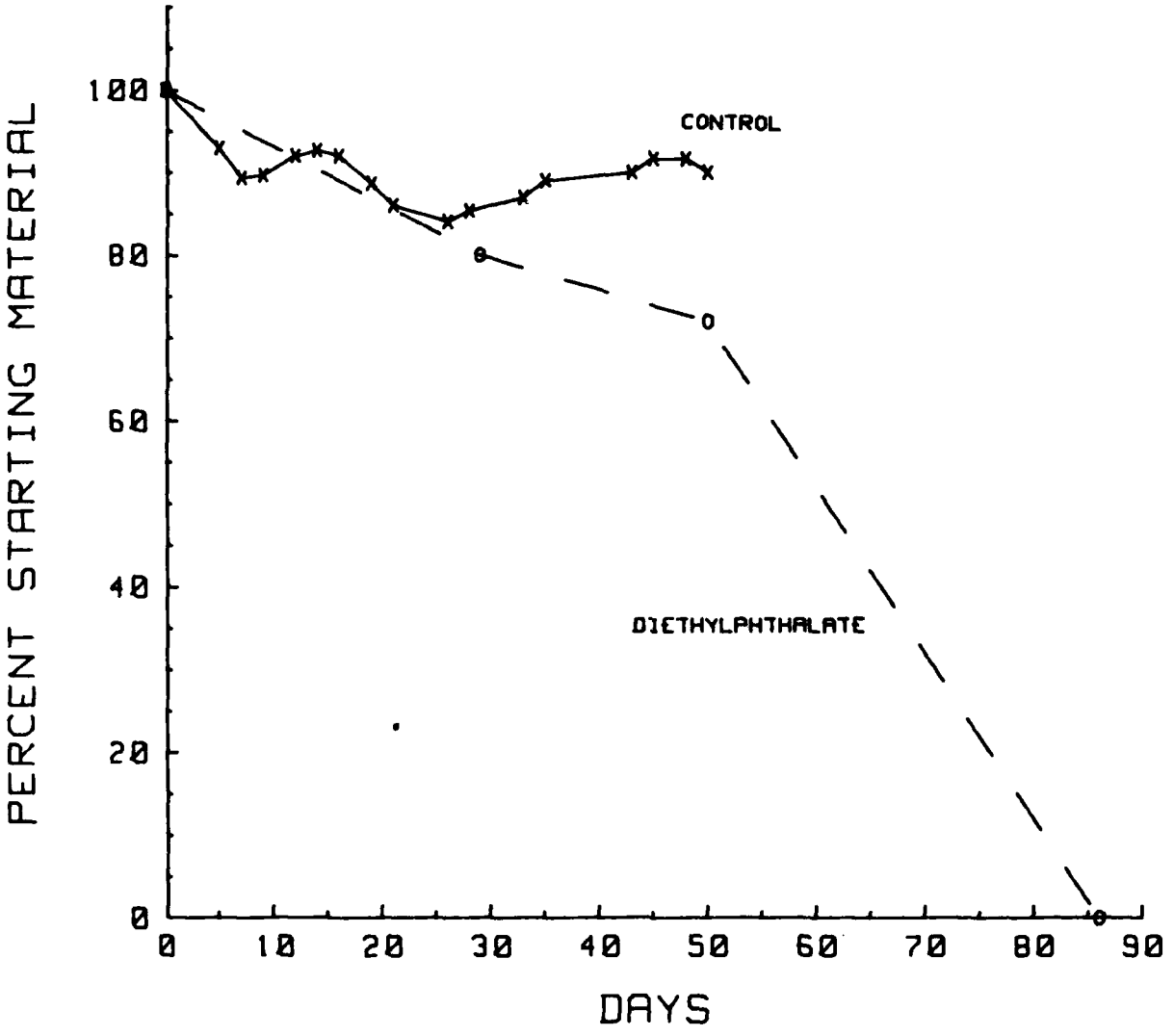


Figure 5. Disappearance of diethylphthalate in a static culture under anaerobic conditions.

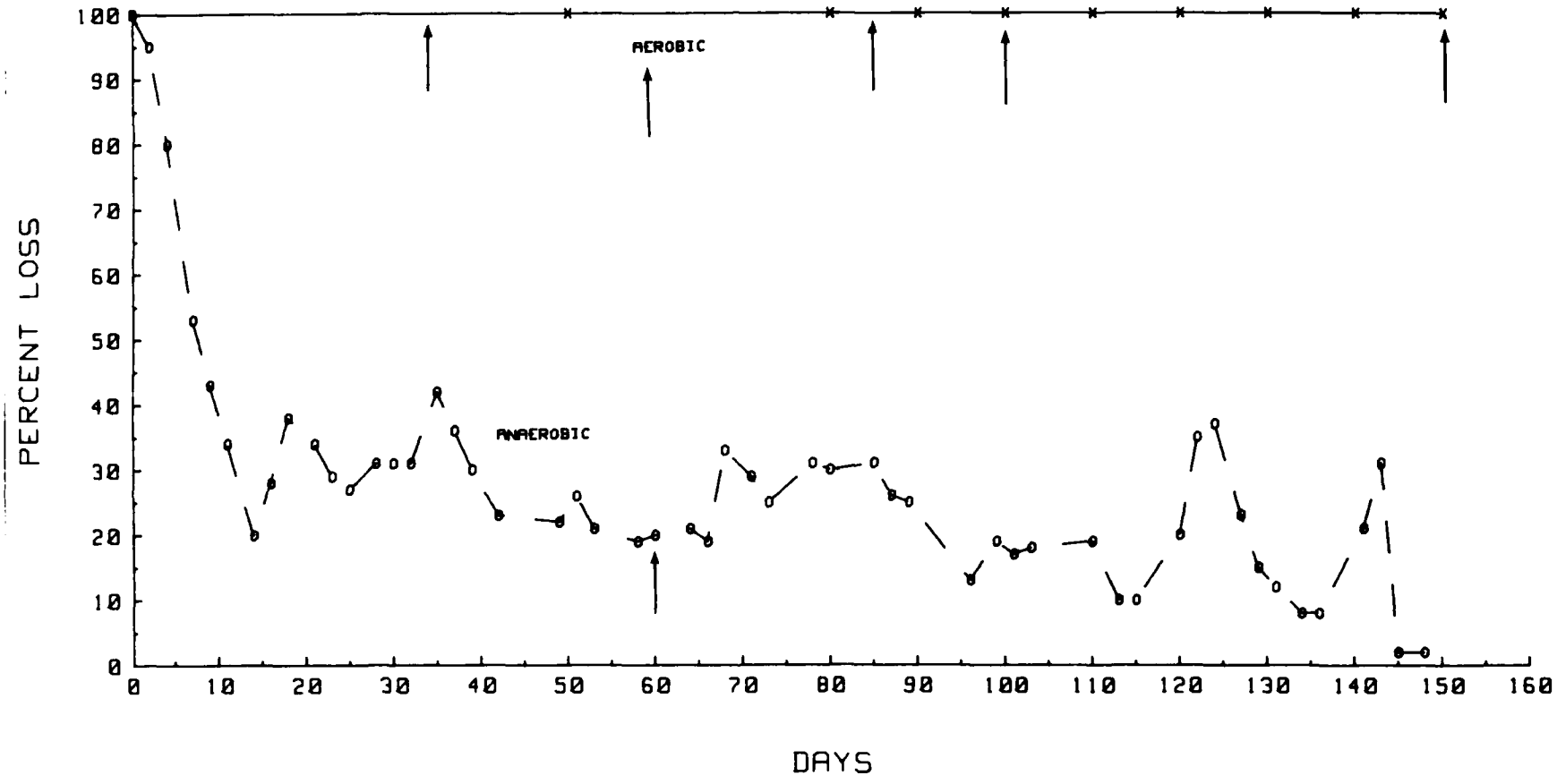


Figure 6. Disappearance of diethylphthalate in a continuous culture system.

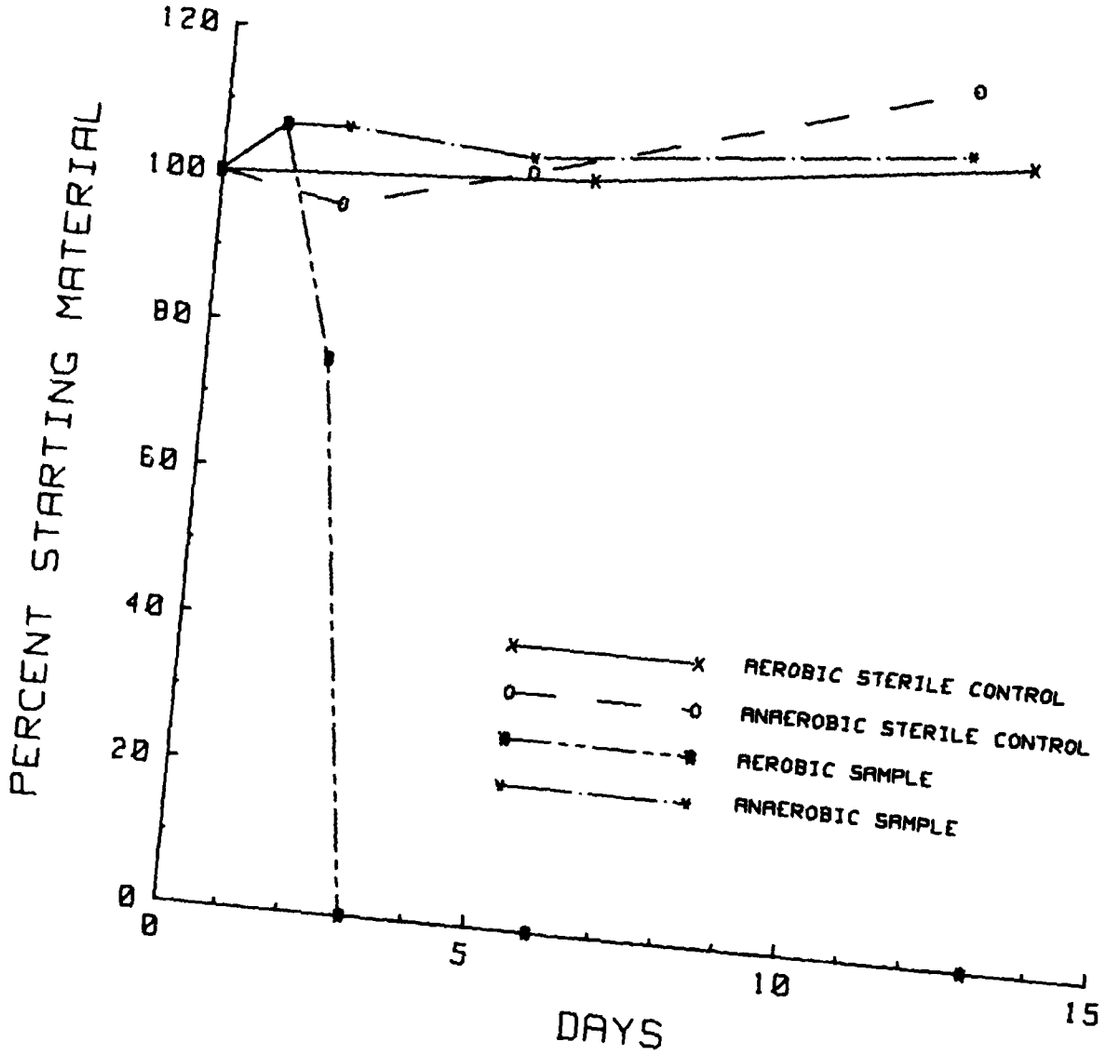


Figure 7. Disappearance of 2-ethylhexanoic acid in a static culture.

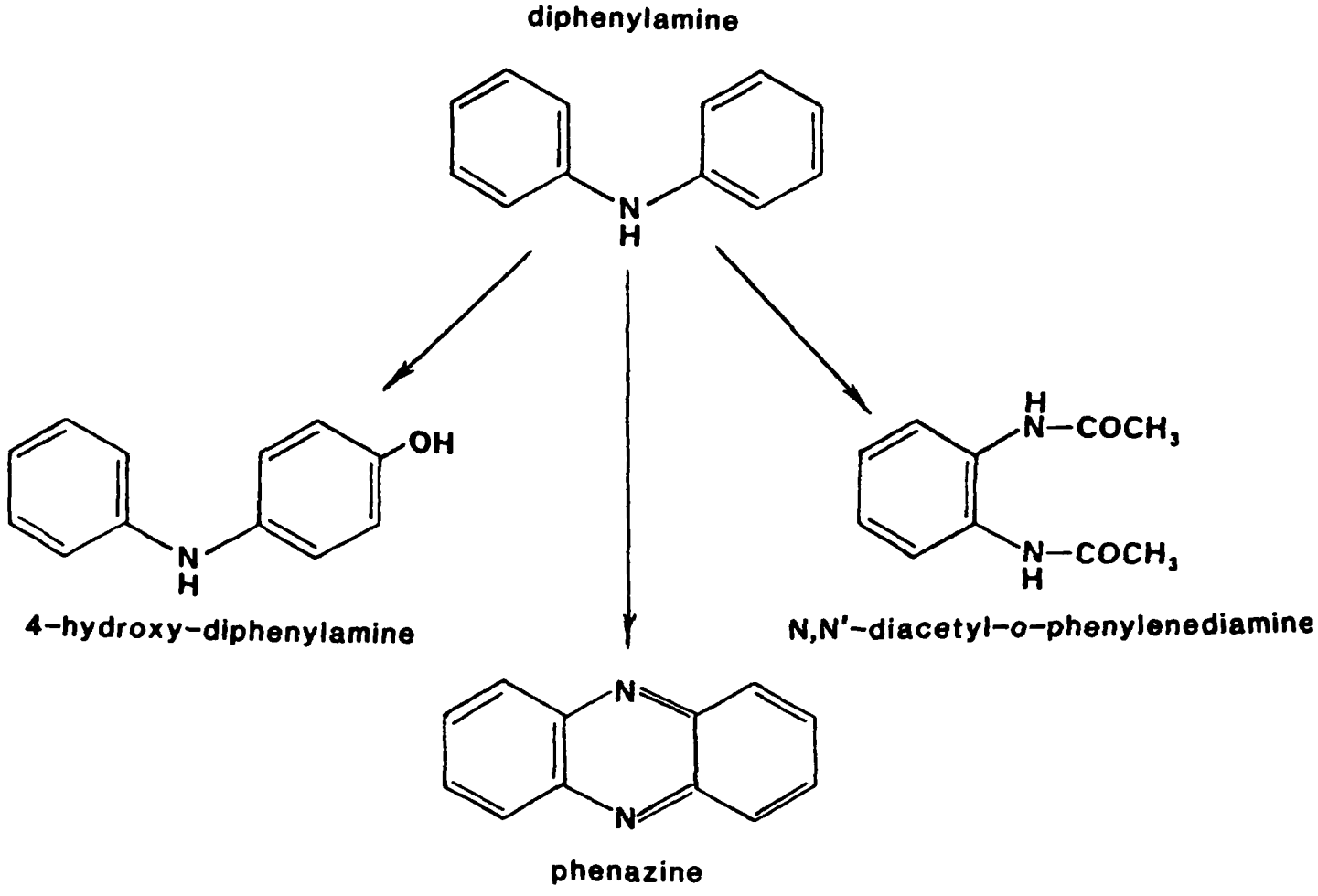


Figure 8. Metabolic pathway for production of biotransformation products from diphenylamine.

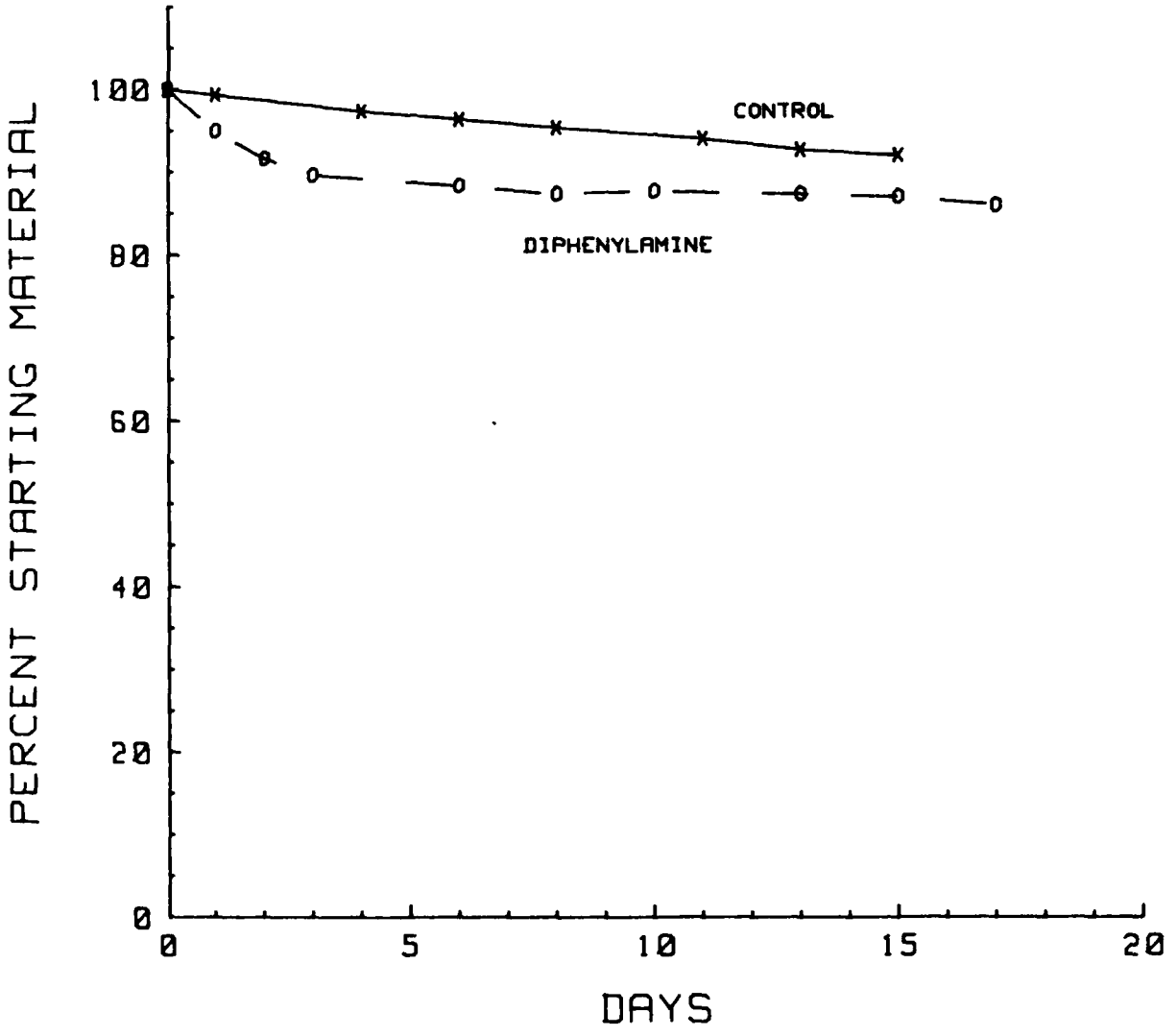


Figure 9. Disappearance of diphenylamine in a static culture under anaerobic conditions.

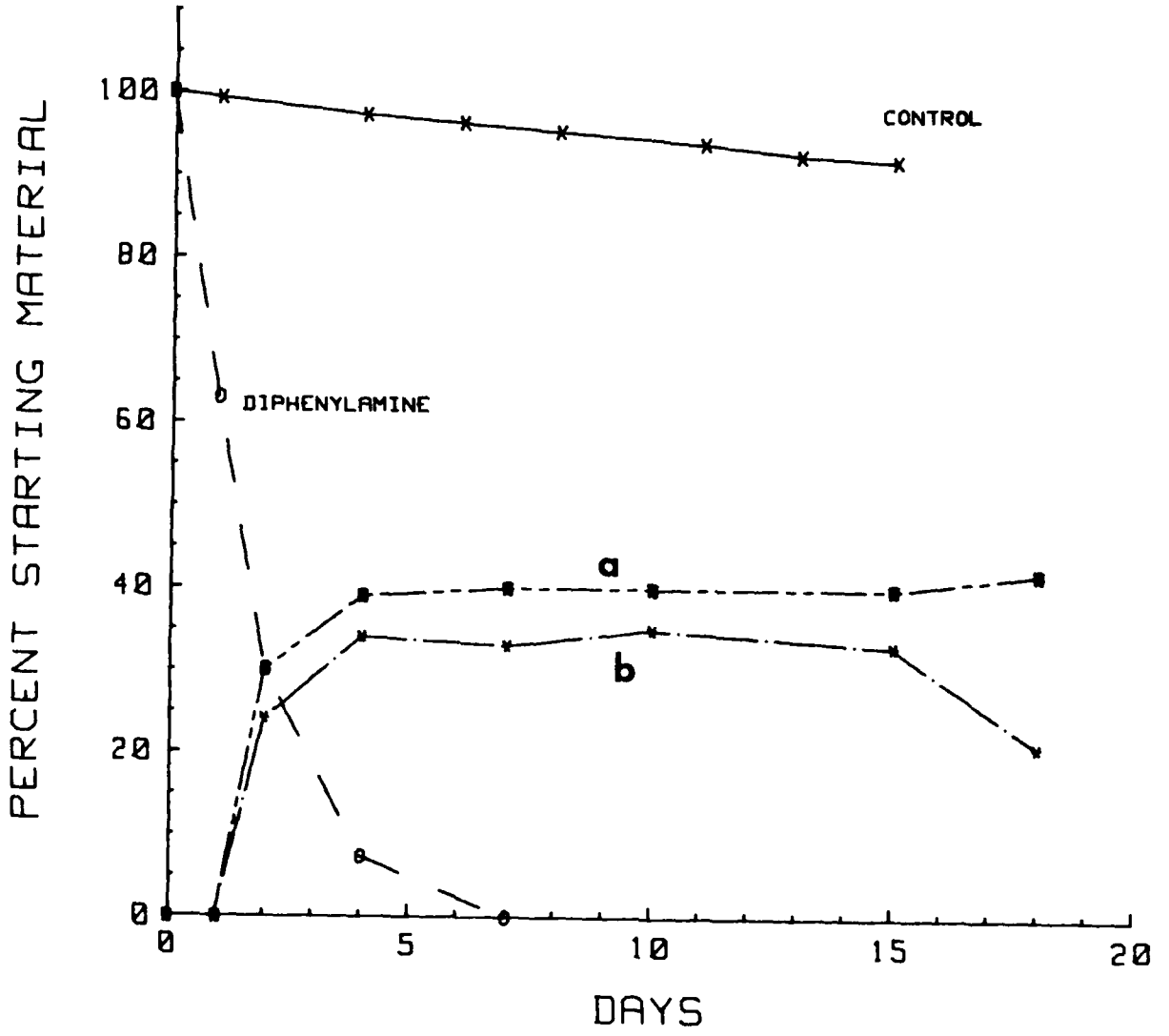


Figure 10. Disappearance of diphenylamine in a static culture under aerobic conditions; a = 2.8 min intermediate, b = 3.2 min intermediate.

extracts from residual DPA is illustrated in Fig. 11. The samples were extracted with methylene chloride and the extract concentrated 1000-fold.

The separation shown is that achieved using a "semi-prep" column; thus, the times of elution are somewhat greater than when using an analytical column, but larger quantities of material could be purified and separated. The residual DPA which was not detectable after 7 days (Fig. 9) now becomes visible after concentration of the sample. The DPA and the two intermediates account for less than 0.1% of the starting material.

The disappearance of DPA in an aerobic continuous culture is illustrated in Fig. 12. The initial retention time was four days and the medium consisted of nutrient broth (8 g/L). At day 11 the concentration of the medium was lowered to 10% of full-strength nutrient broth (0.8 g/L) and after a few days of acclimation the disappearance again approached 100%. The nutrient medium was changed again at day 35 to 0.4 g/L of nutrient broth, and at day 59 to a basal salts medium in distilled water. Efficiency of breakdown was reduced when the only available carbon source was DPA. Each peak and trough in the curve after day 94 indicates a change in effective retention time. A four day retention time resulted in a decrease to 80% disappearance while increasing the retention time to 6 days increased the efficiency to nearly 100% disappearance.

The chromatogram in Fig. 13 is of a sample from an aerobic continuous culture of DPA from which two liters of effluent were collected over a period of two to three weeks. The sample was centrifuged, extracted and concentrated 2000-fold. In this concentrated sample several intermediates were separated and identified. The compounds corresponding to the labeled peaks were identified by comparison with the properties of known reference compounds with respect to elution times, with both analytic and "semi-prep" HPLC. Analysis by GC/MS was also used to confirm identities. These intermediates were detected only after concentration of several thousand-fold; hence, are present in the effluent at very low concentrations.

2-Nitrodiphenylamine (2NDPA). The structures of this compound and of several breakdown products are depicted in Fig. 14. The formation of 2-aminodiphenylamine presumably takes place under anaerobic conditions through reduction of the nitro group. N-phenylbenzimidazole and phenazine were also detected under both aerobic and anaerobic conditions. Phenazine was found to be a contaminant in our 2NDPA standard, which only became apparent after concentration of the samples. The concentration of phenazine in our samples was higher than that which would be expected from a similar concentration of the standard 2NDPA, which indicates that phenazine was formed in small amounts in the experimental cultures and then slowly degraded.

The aerobic disappearance of 2NDPA is shown in Fig. 15. The initial concentration was 10 ppm. After an initial burst of activity the rate of disappearance dropped off considerably. The medium used in this experiment was 0.8 g/L of nutrient broth and the resulting degradation approached 60% after two weeks. Similar experiments which had a higher concentration of

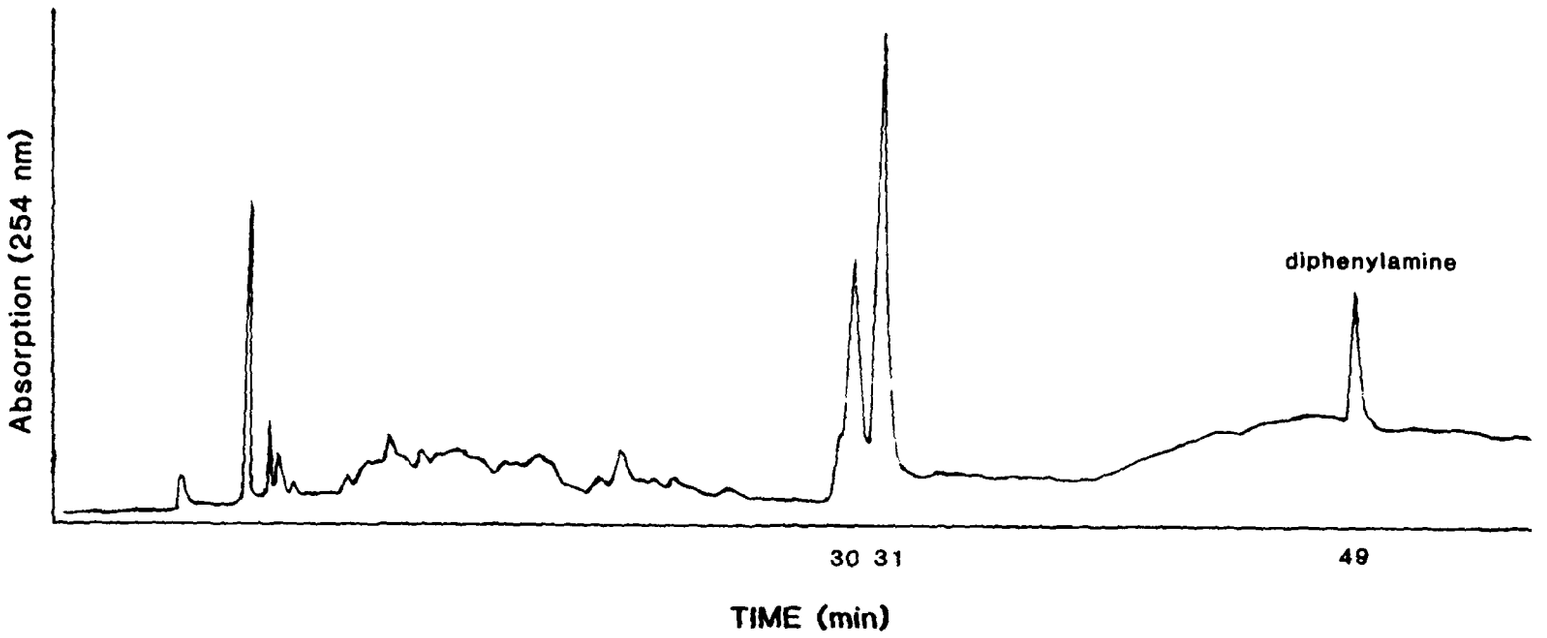


Figure 11. HPLC analysis of concentrated extracts of a static culture containing diphenylamine and incubated under aerobic conditions.

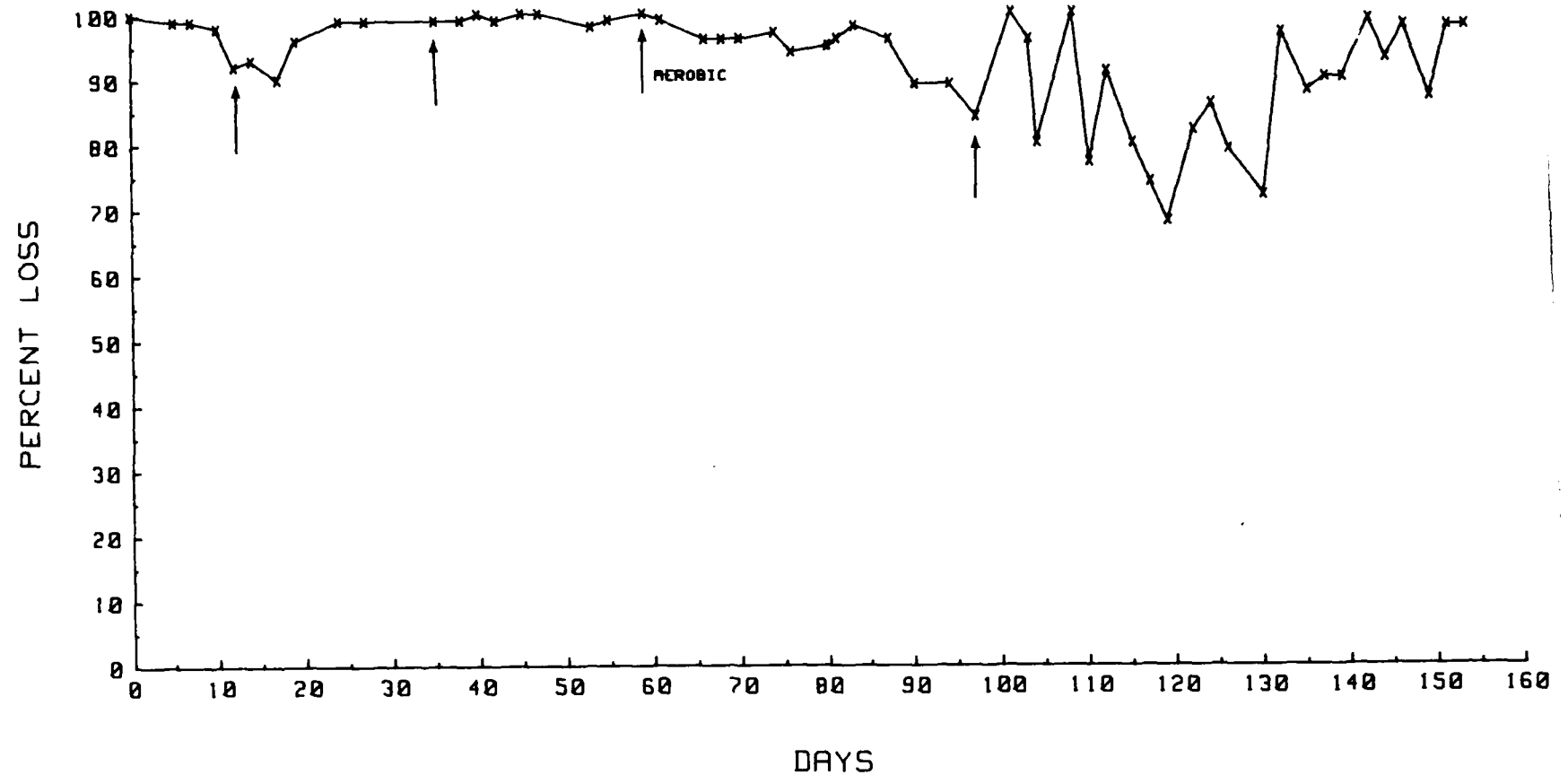


Figure 12. Disappearance of diphenylamine in a continuous culture system under aerobic conditions.

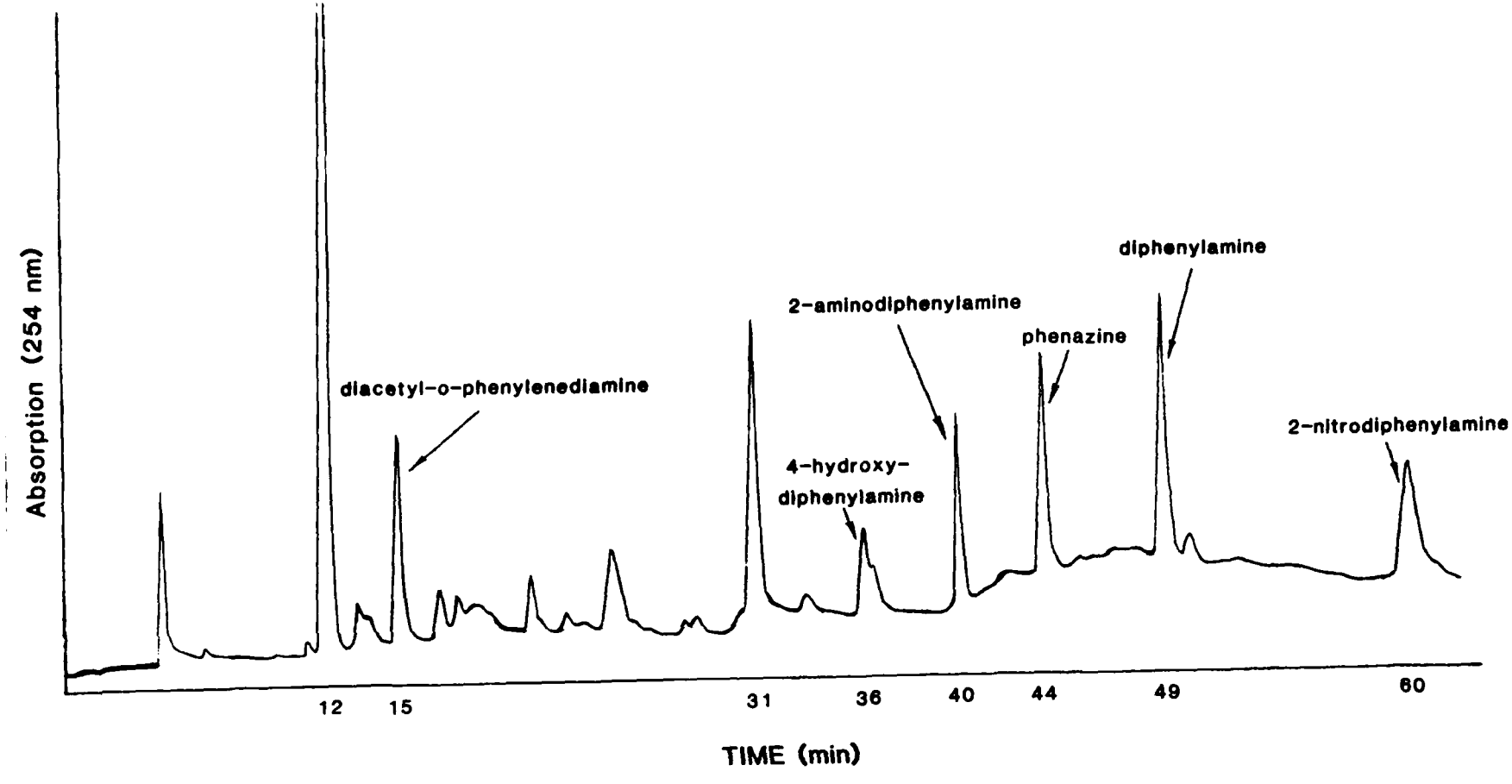


Figure 13. HPLC analysis of concentrated extracts of effluents from a continuous culture system containing diphenylamine and incubated under aerobic conditions.

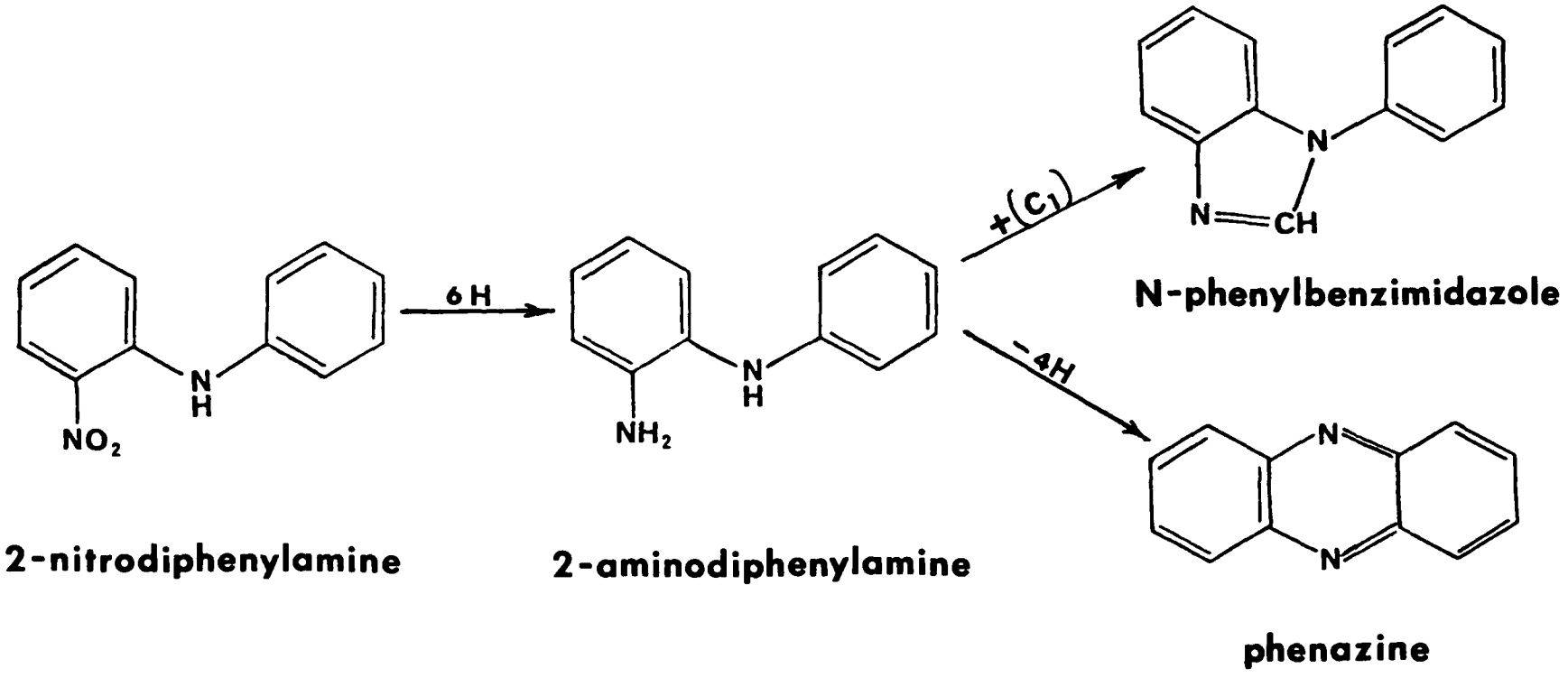


Figure 14. Metabolic pathway for production of biotransformation products from 2-nitrodiphenylamine.

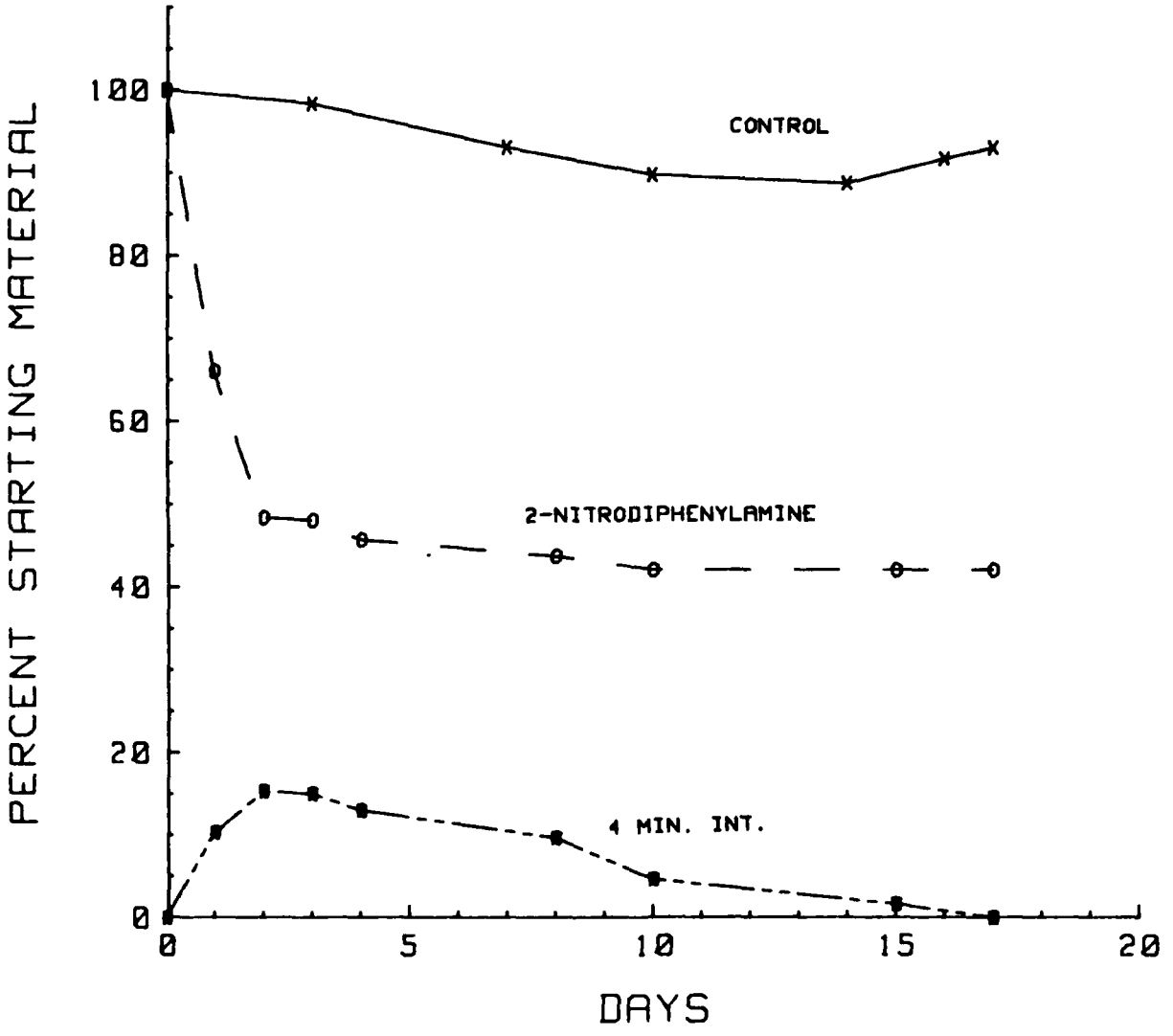


Figure 15. Disappearance of 2-nitrodiphenylamine in a static culture under aerobic conditions.

available carbon (i.e., full strength nutrient broth, 8 g/L) resulted in faster and more efficient degradation approaching 90%. The curve labeled "4 min. int." represents the appearance and subsequent degradation of the aggregate intermediates, which remained unresolved on the analytical column but separated into three major isolated peaks on the "semi-prep" column. Figure 16 shows the intermediates found in the aerobic batch cultures of 2NDPA after concentration and HPLC analysis on the "semi-prep" column. Without concentration of the sample the only compound present in high enough concentration to be detected would be the residual 2NDPA, seen as a very large peak at 60 minutes. The area of this peak represents the 4 ppm of 2NDPA which remained after 17 days. In this experiment the level of intermediates totaled somewhat less than 1% of the starting concentration of 2NDPA.

Under anaerobic conditions 2NDPA disappeared rapidly from static cultures (Fig. 17) in a medium composed of 0.8 g/L of nutrient broth. After three days no 2NDPA (initial concentration, 10 ppm) could be detected. As with aerobic cultures, a single aggregate curve which is labeled "4 min. int." appeared. This material was resolved into several individual peaks with the "semi-prep" column as shown in Fig. 18. No 2NDPA was detected but 2-aminodiphenylamine, phenazine and N-phenylbenzimidazole were identified.

The results obtained from aerobic and anaerobic continuous culture systems in a nutrient broth (4 g/L) medium are shown in Fig. 19. Under aerobic conditions, there was 100% disappearance at a retention time of five days. The arrow (a) at 37 days indicates the point at which the nutrient broth concentration was reduced to 0.4 g/L. The percent loss started to decline as degradation became less efficient. At day 77 (arrow b) the concentration of nutrient broth was increased to 0.8 g/L, doubling the carbon source, and the retention time was reduced to four days. Under these conditions the percent loss dropped, so the retention time was increased to five days and the system started to show better activity. Each of the peaks and troughs after 90 days represents changes in retention time and illustrates the sensitivity of such systems to the flow-rate of influent. No intermediates were detected in unconcentrated samples. After extraction and concentration the same intermediates were identified as found in static cultures (Figs. 16 and 17).

In the anaerobic continuous culture system (Fig. 19) the initial nutrient concentration was 4 g/L of nutrient broth. At day 28 (arrow c) the nutrient broth was reduced to 0.4 g/L and the efficiency of the system dropped to less than 10% loss. At day 67 (arrow d) the nutrient broth concentration was doubled to 0.8 g/L and shortly after that the percent loss rose to 100%. The influent in all anaerobic continuous culture experiments contained 500 ppm of nitrate, and in all cases, no nitrate was detected in the effluent, indicating complete denitrification.

Centralite (Diethyldiphenylurea). The structures of centralite and of several metabolic intermediates are shown in Fig. 20. In a static culture under aerobic conditions, centralite disappeared rapidly at first and then tailed off into a much slower disappearance (Fig. 21). The decrease in centralite was accompanied by the appearance of two intermediate peaks, one at 3.7 minutes which was identified as N-ethylaniline and one at 4.6 minutes

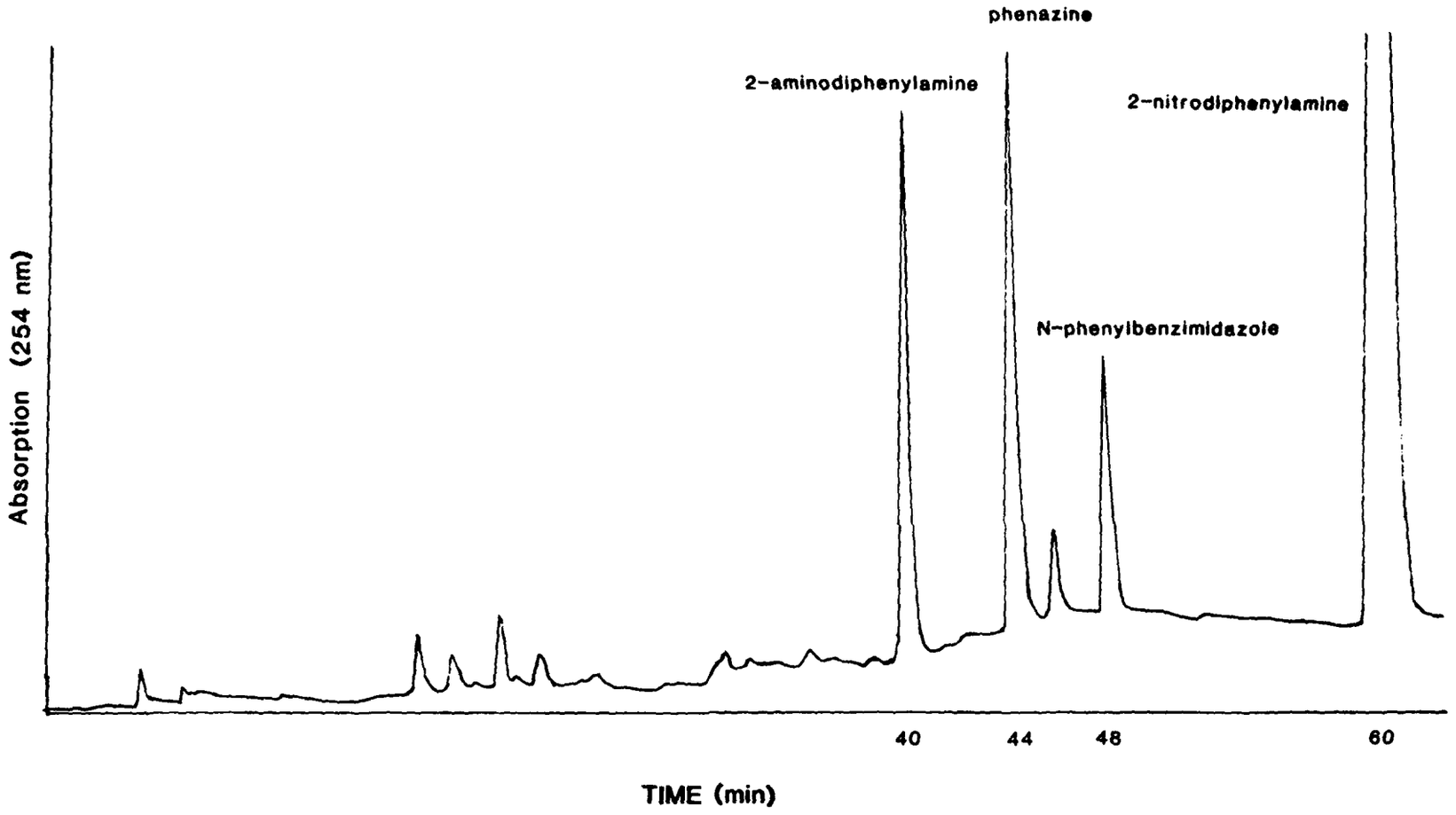


Figure 16. HPLC analysis of concentrated extracts from a static culture containing 2-nitrodiphenylamine and incubated under aerobic conditions.

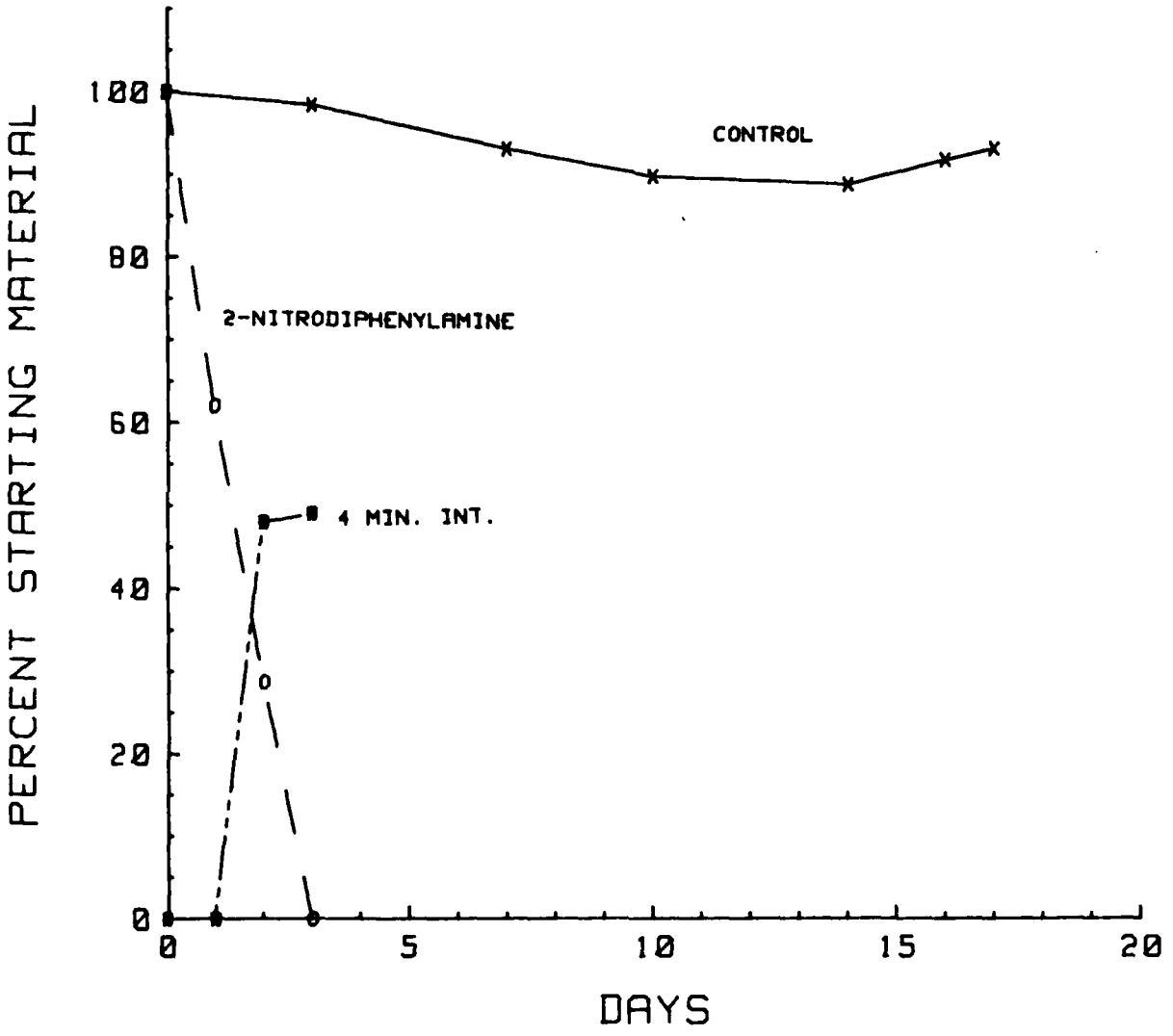


Figure 17. Disappearance of 2-nitrodiphenylamine in a static culture under anaerobic conditions.

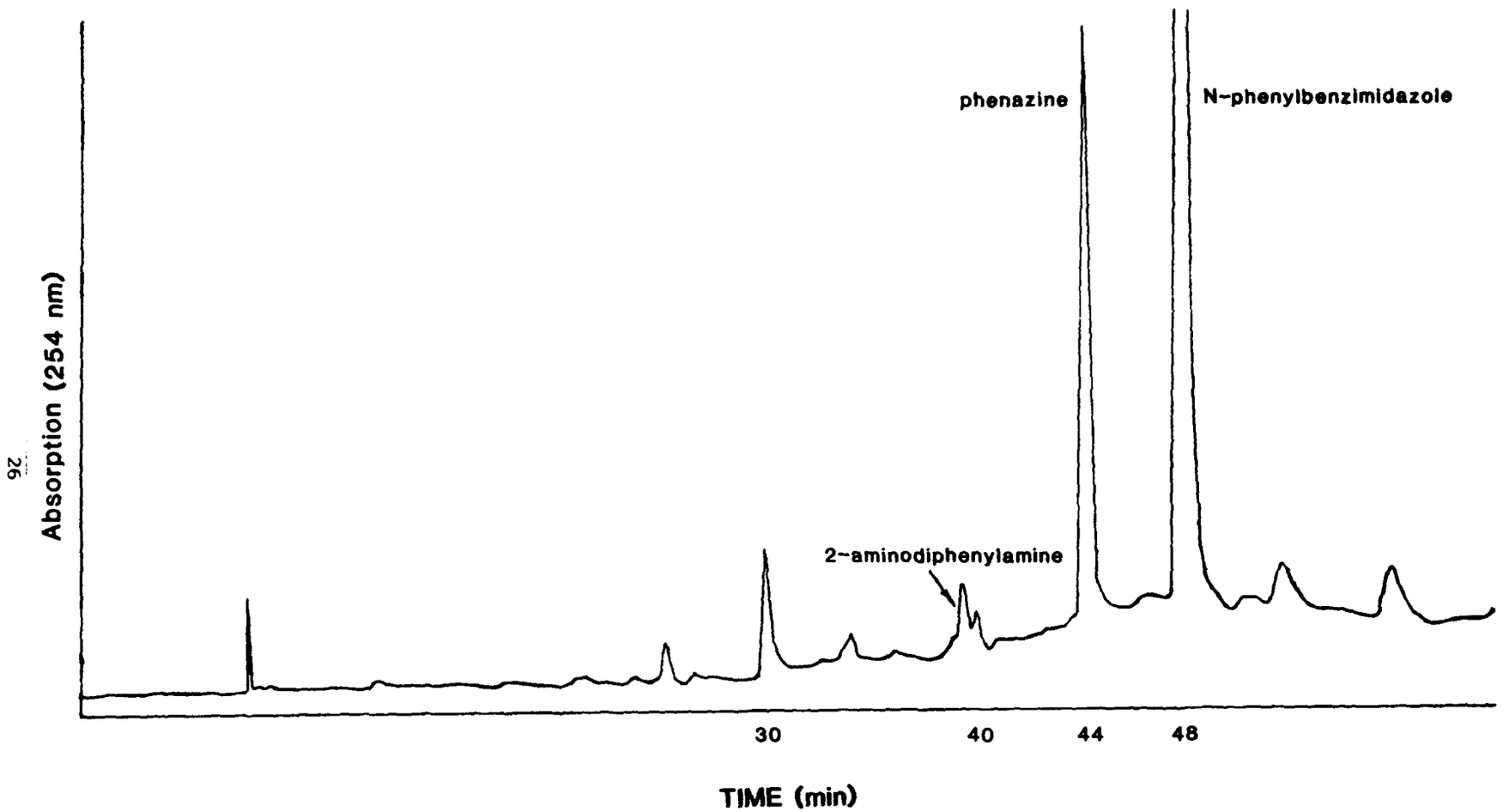


Figure 18. HPLC analysis of concentrated extracts from a continuous culture system containing 2-nitrodiphenylamine and incubated under anaerobic conditions.

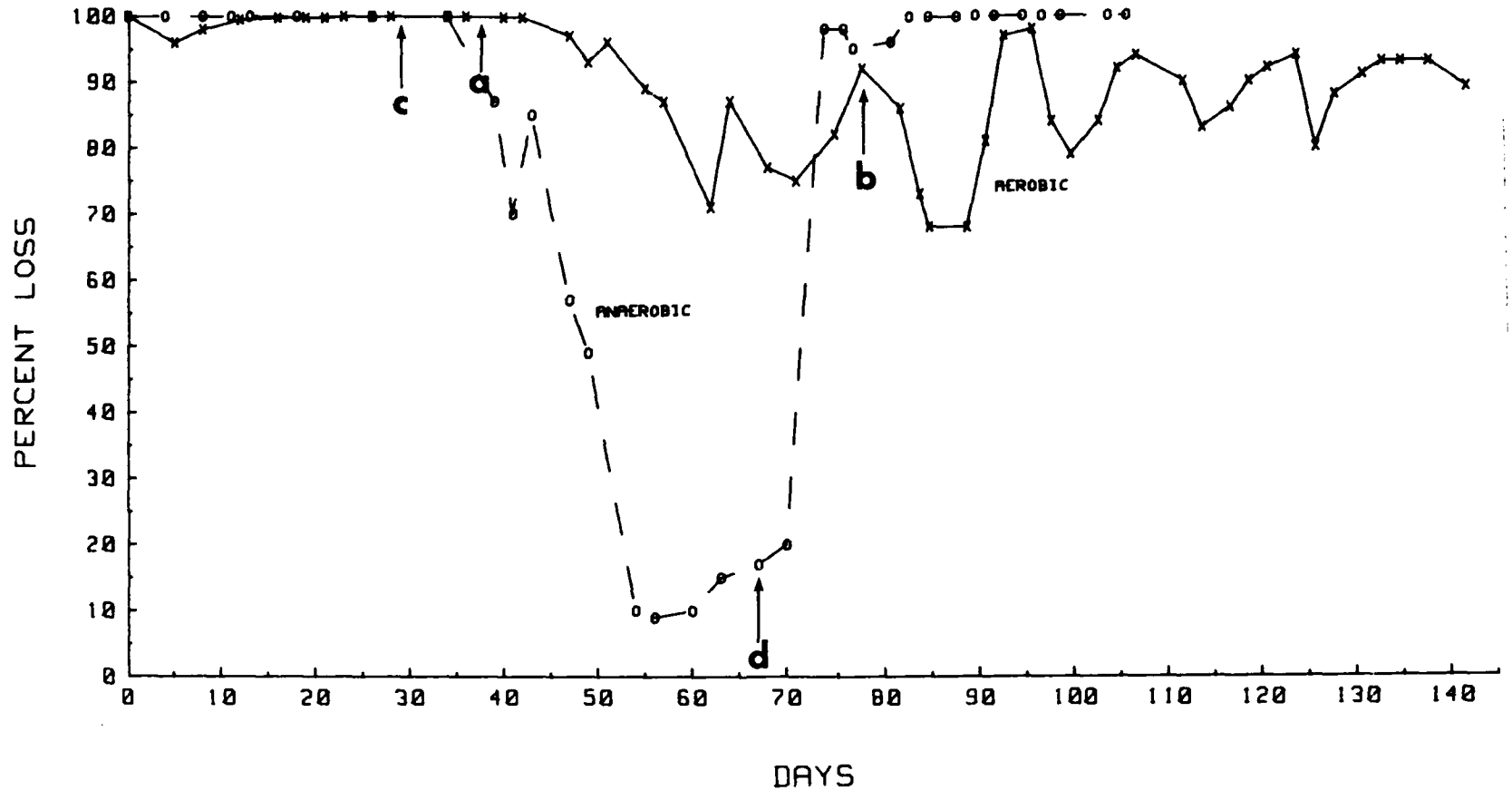


Figure 19. Disappearance of 2-nitrodiphenylamine in a continuous culture system.

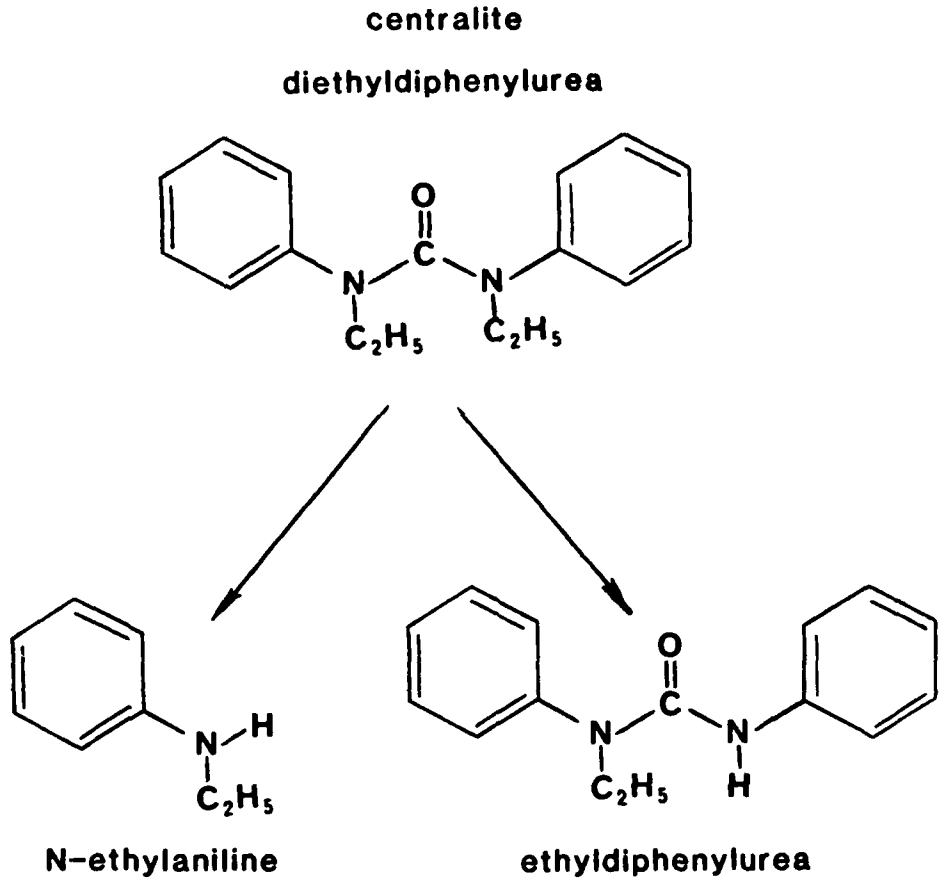


Figure 20. Metabolic pathway for production of biotransformation products from centralite.

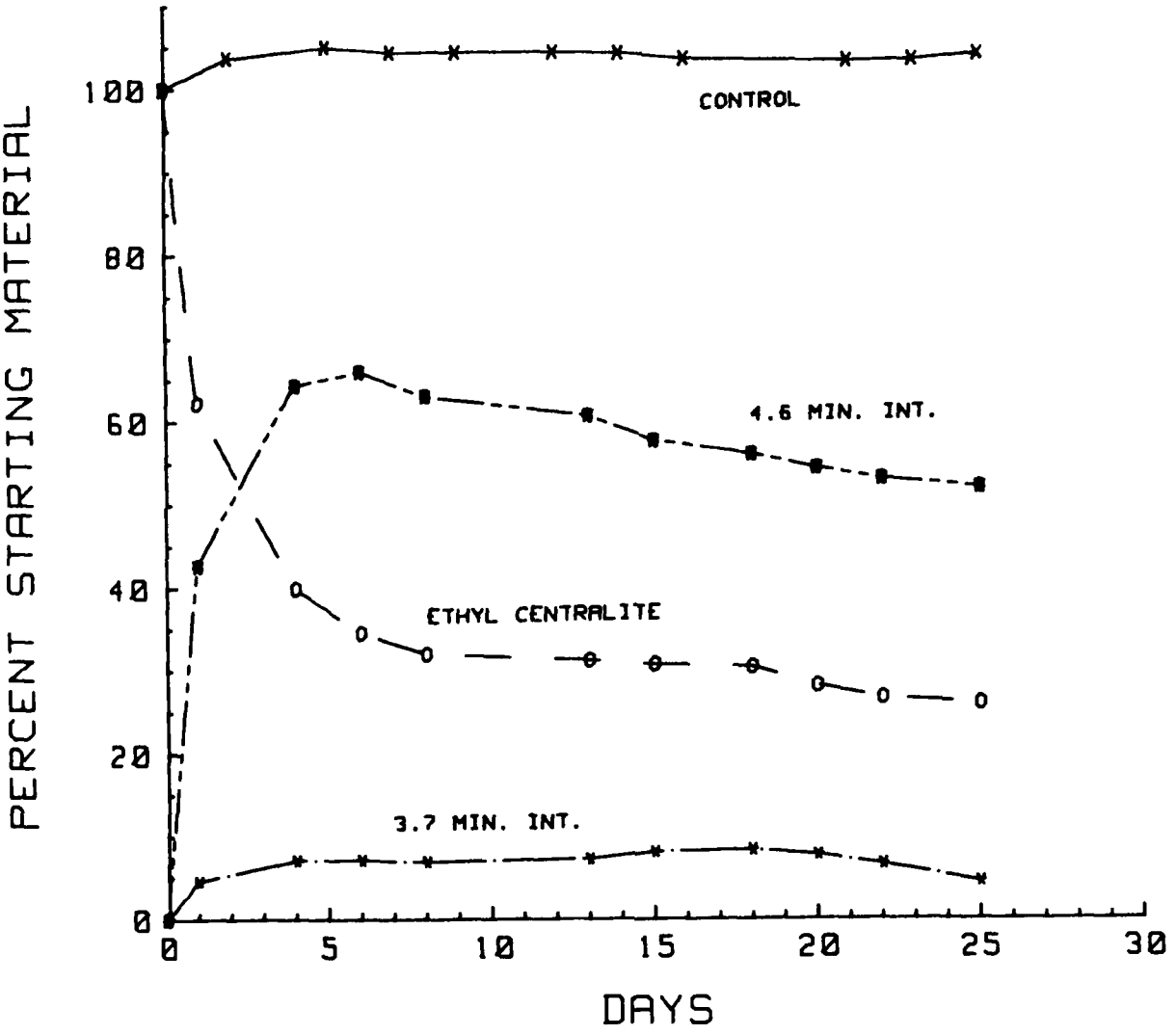


Figure 21. Disappearance of centralite in a static culture under aerobic conditions.

which was identified as ethyldiphenylurea. The identities were confirmed by GC/MS analysis. Centralite appears to be quite stable to anaerobic degradation. Figure 22 shows the behavior of such a system in a rich medium (8 g/L of nutrient broth).

The results obtained from aerobic and anaerobic continuous cultures containing 10 ppm of centralite are shown in Fig. 23. The aerobic vessel was started with a retention time of 5 days and a nutrient medium of 0.8 g/L of nutrient broth. At day 106 (arrow) the system was switched to 1.6 g/L of nutrient broth but the efficiency did not improve. The flow was stopped at day 141 and within 5 days the efficiency increased to 80%. At this point there was no centralite feeding in so the remaining centralite was being degraded as if it were in a static culture.

The anaerobic continuous culture maintained an E_h of -280 to -300 mV throughout the experiment. At day 36, 500 ppm of nitrate were added to the influent feed and within 5 days no nitrate could be detected in the effluent. The anaerobic continuous culture system appeared much more active toward centralite than the anaerobic batch (static) system.

2,4-Dinitrotoluene. Some of the expected biotransformation products from 2,4-DNT are separated by HPLC by using 40% methanol in water as the mobile phase at a flow-rate of 2 mL/minute and detected at 230 nm (Fig. 24).

A continuous culture was set up using lake water, basal salts, and methanol. HPLC analysis of effluent showed the disappearance of 2,4-DNT and the appearance of 2-amino-4-nitrotoluene (Fig. 25, peak a) and 4-amino-2-nitrotoluene (Fig. 25, peak b). When the methanol concentration was reduced to 0.7 mg/L the intermediates disappeared. When the methanol was cut out completely the intermediates reappeared (Fig. 25, peaks a and b) and the efficiency of 2,4-DNT removal dropped to 90% (Fig. 25, peak c). The system was next altered by substituting distilled water for lake water. The efficiency dropped further to 79% removal of 2,4-DNT (Fig. 25, peak c), while the level of intermediates rose (Fig 25, peaks a and b).

Two continuous culture systems were set up using ^{14}C-labeled 2,4-DNT (total 2,4-DNT concentration was 10 mg/L) in a nutrient broth medium. One was set up aerobically and the other anaerobically. The anaerobic system contained 0.04 M KNO_3 and was monitored for denitrification efficiency. Throughout the course of the experiment >99% denitrification was observed. The distribution of radioactivity among various fractions is reported in Table 3. In the anaerobic system, most of the radioactivity was present in the fraction subjected to HPLC analysis. With the aerobic system, considerable radioactivity was lost during the evaporation of methylene chloride from the extract, for a recovery of little more than 25% of the total counts for use in HPLC analysis. The loss in counts is unexplained since the concentration procedures were very mild (i.e., evaporation at 50°C under a stream of N_2).

TABLE 3. Fractionation of Radioactivity from ^{14}C-Labeled 2,4-DNT Incubated under Aerobic and Anaerobic Conditions in Continuous Culture Systems

Fraction tested	Percent Recovery[a]	
	Aerobic	Anaerobic
Initial	100	100
Pooled effluents	90[b]	83
Methylene chloride extraction	99	99
Concentration	38	99
HPLC fractions	82	83

[a]Reported as recovery from preceding treatment, not total recovery.

[b]An additional 0.2% was found in the alkaline trap, 0.9% in the acid trap, and 0.2% in the water trap.

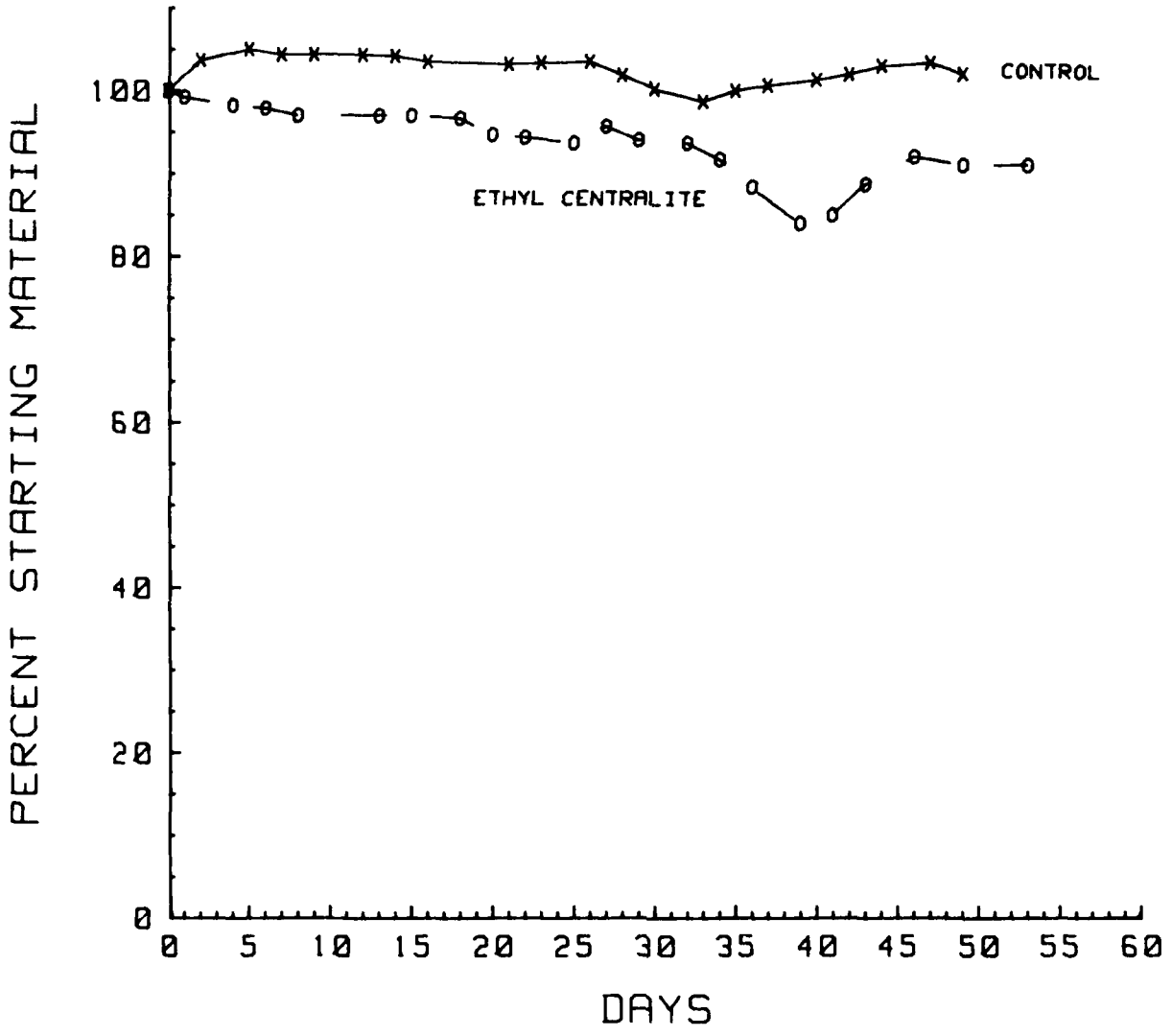


Figure 22. Disappearance of centralite in a static culture under anaerobic conditions.

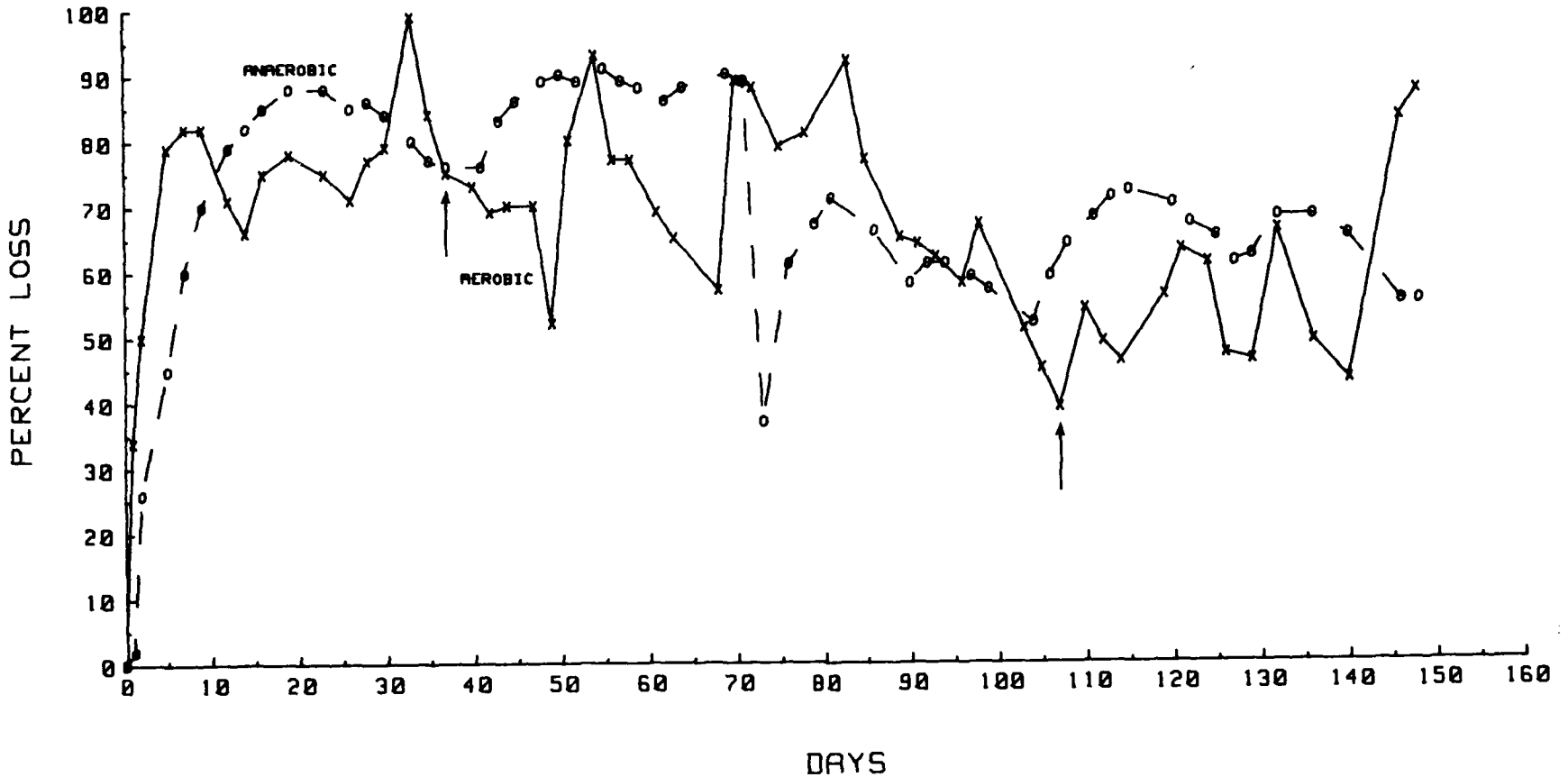


Figure 23. Disappearance of centralite in a continuous culture system.

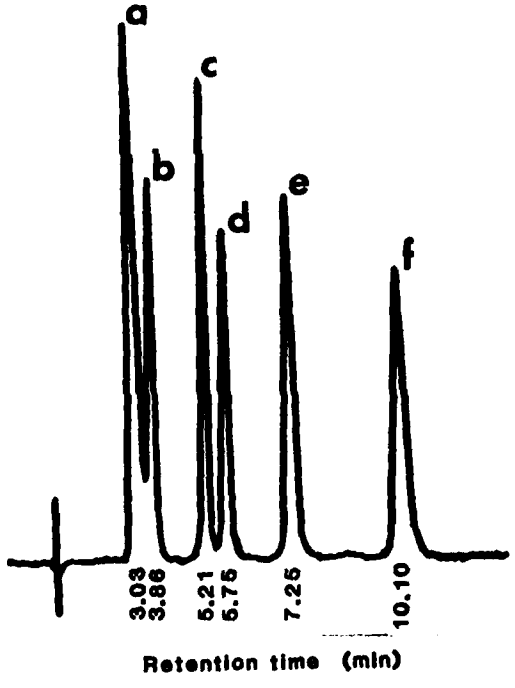


Figure 24. HPLC analysis of a mixture of compounds involved in biotransformation reactions of 2,4-DNT: a = 2,4-diaminotoluene; b = 2-acetamido-4-nitrotoluene; d = 2-amino-4-nitrotoluene; e = 4-acetamido-2-nitrotoluene; f = 2,4-dinitrotoluene.

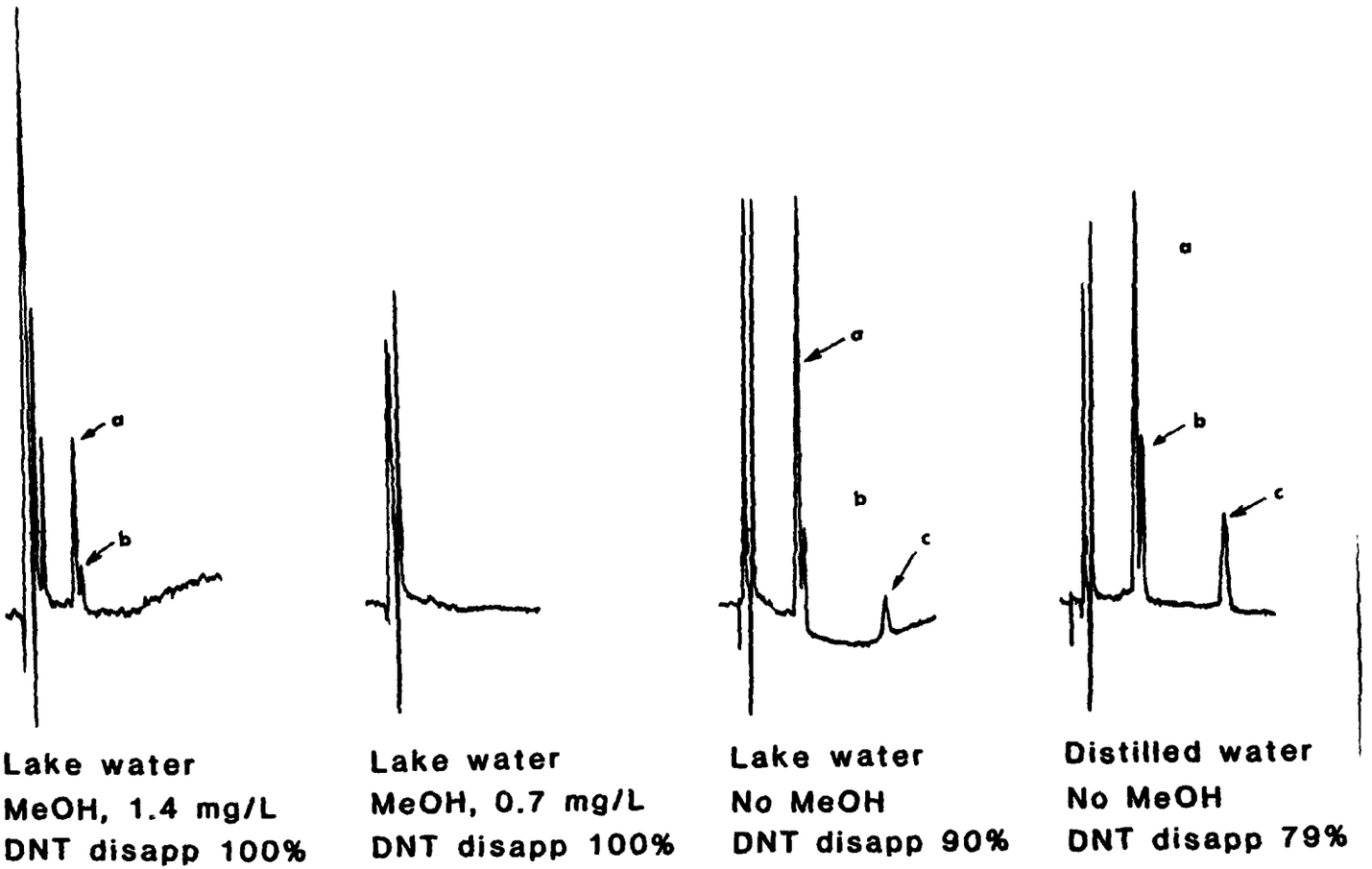


Figure 25. Effect of composition of nutrient medium on the fate of 2,4-DNT in an aerobic continuous culture system.

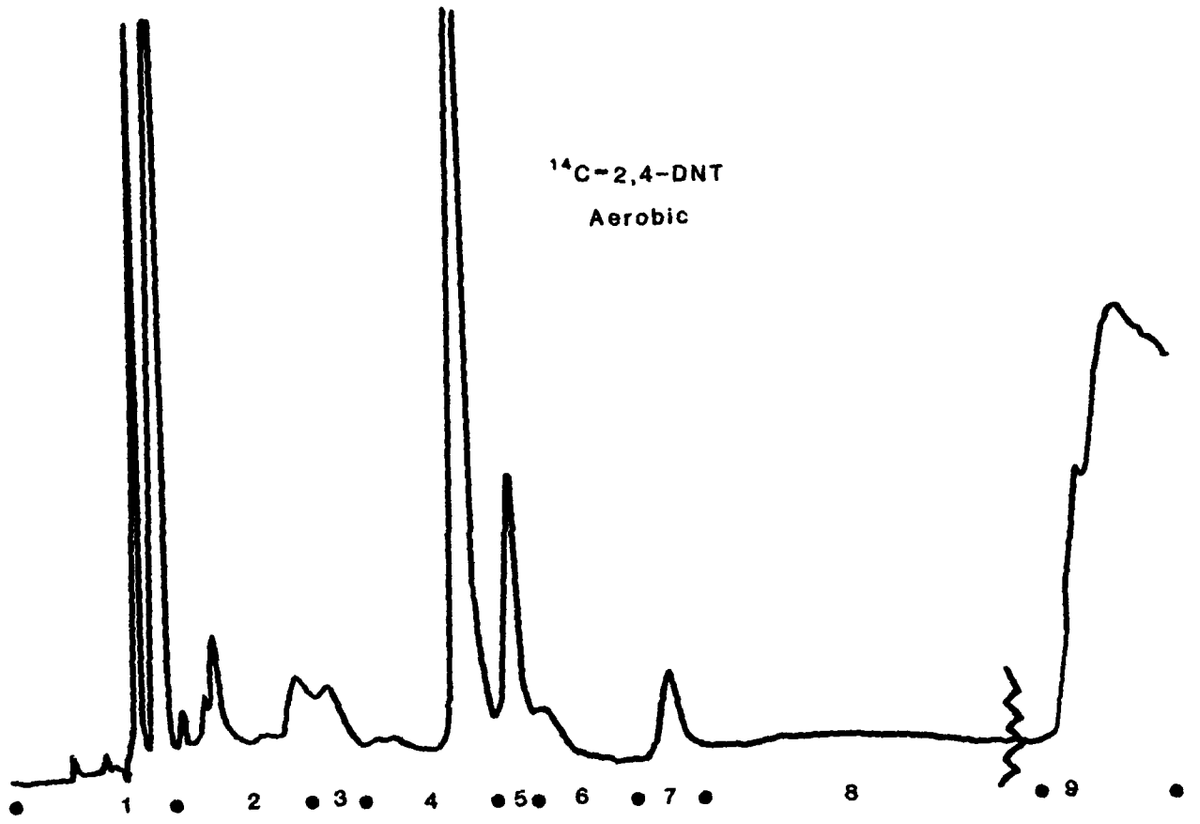


Figure 26. HPLC separation of extracts from an aerobic biotransformation of ^{14}C-labeled 2,4-DNT.

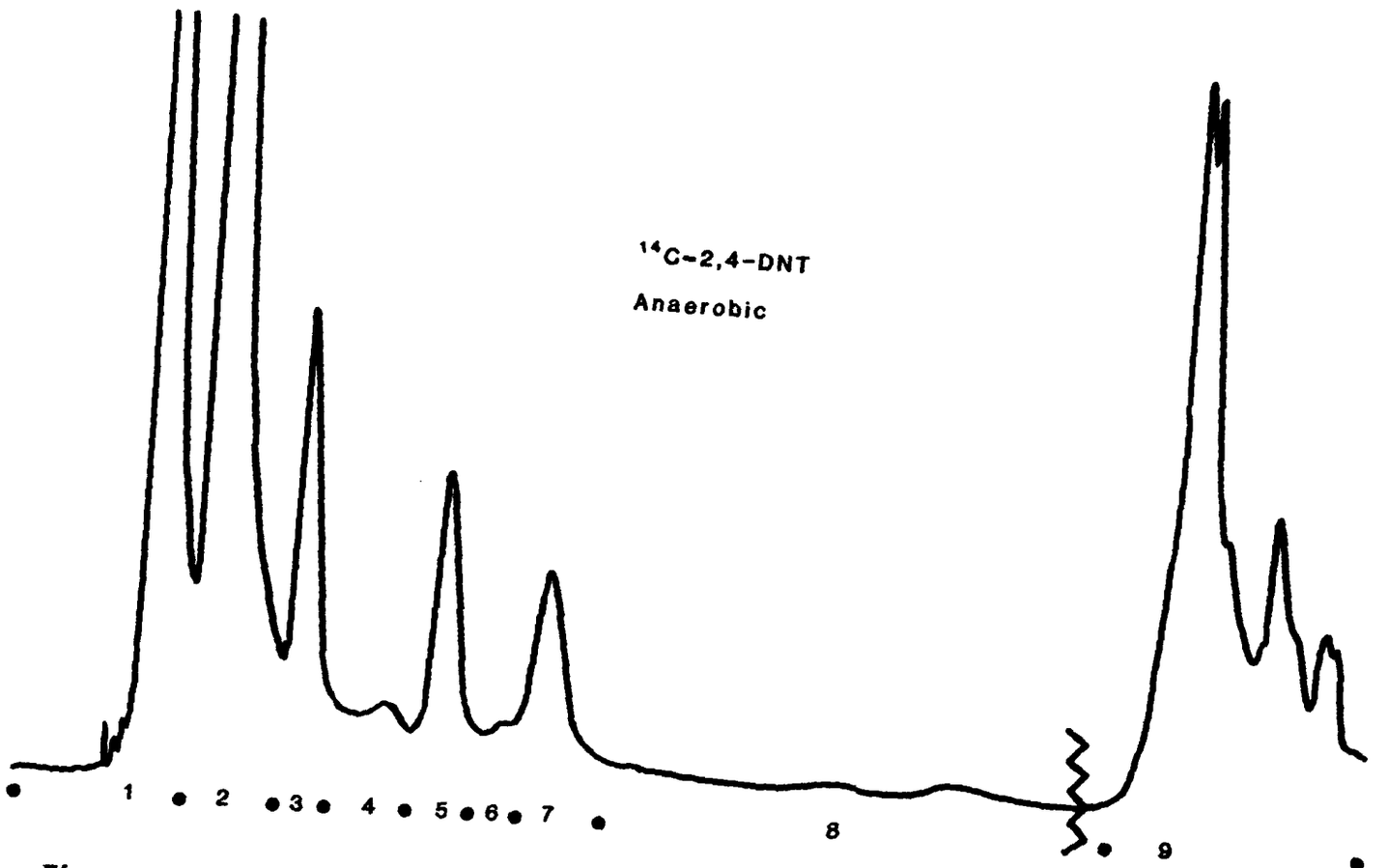


Figure 27. HPLC separation of extracts from an anaerobic biotransformation of ^{14}C-labeled 2,4-DNT.

Table 4 shows the recovery of counts in various peaks after HPLC analysis (as percent of the amount placed on the column). Peaks 4, 5, and 7 of the aerobic system (Fig. 26) were identified as 4-amino-2-nitrotoluene, 2-amino-4-nitrotoluene, and 4-acetamido-2-nitrotoluene, respectively. The radioactivity-containing peaks from HPLC analysis of the anaerobic system (Fig. 27) did not match up with any of the known reference compounds examined, and remain unidentified.

DISCUSSION

The results reported here, in agreement with many other reports, show that the phthalate esters are completely broken down whether incubated under aerobic or anaerobic conditions. On the other hand no disappearance of 2-ethylhexoate was noted under anaerobic conditions but aerobically the compound disappeared rapidly, in static or continuous culture.

Diphenylamine disappeared rapidly from aerobic static cultures with the formation of several intermediates. No DPA disappearance was detected in anaerobic cultures either in static or continuous culture systems. No additional intermediates were detected in these batch cultures after extraction and concentration of several thousand-fold. However, after concentration of pooled effluents from aerobic continuous cultures many intermediates were evident. Further work would be required to ascertain the fate of some of these intermediate compounds. The identification of N-phenyl benzimidazole as a transformation product of 2-nitrodiphenylamine is the first report of the synthesis of this compound in a biological system. Not only is the nitro compound reduced to an amino compound, but a C_1 fragment from the metabolic pool is incorporated to form a third ring.

Ethylcentralite was expected to be easily biodegradable and the intermediates detected suggest that this is the case. If the work had been continued, it is probable that further loss of the remaining phenyl and ethyl groups would have been observed, followed by complete degradation of the resulting residues. The attack on the centralite molecule appears to be strictly oxidative since no activity was observed in anaerobic batch cultures. The small amount of activity observed in anaerobic continuous culture is not understood at this time.

The results obtained with 2,4-DNT in continuous cultures fed lake water supplemented with methanol showed that the expected reduction products were formed. The disappearance of these products from the effluent upon decreasing the methanol concentration may be a rate-related phenomenon. Perhaps under reduced levels of available carbon, most of the products went into cell mass and thus the biotransformation of 2,4-DNT continued to be 100%. When methanol was eliminated completely from the system, the thick cellular mat adhering to the sides of the culture vessel, which had been building up during several months of operation, evidently contained a sufficiently active consortium of microorganisms to biotransform 2,4-DNT with the production of the observed intermediates. Even when lake water was replaced by distilled water, sloughing off from the mat was sufficiently slow so that there was still 79%

TABLE 4. Radioactivity from ^{14}C-Labeled 2,4-DNT Recovered after HPLC Fractionation of Concentrated Extracts

	Percent Recovery[a]	
Fraction number	Aerobic	Anaerobic
1	0.9	3.4
2	5.4	38.2
3	0.9	8.2
4	61.3	2.1
5	12.1	5.8
6	1.0	1.2
7	5.2	4.5
8	1.9	3.5
9	11.3	33.2

disappearance of 2,4-DNT accompanied by the appearance of intermediates. Intermediates detected in effluents from anaerobic continuous cultures were different from the amino-nitro-toluenes identified from an aerobic system. These have not been identified.

The ^{14}C-labeling experiments did not support the concept that ring cleavage of 2,4-DNT had occurred, in contrast to the report of Spanggord et al.[9] The amount of ^{14}C trapped as volatile end-products was less than 1%.

CONCLUSIONS

This study has demonstrated that, in the presence of a rich nutrient medium, the compounds diethylphthalate, dibutylphthalate, 2-ethylhexanoate, diphenylamine, 2-nitrodiphenylamine, centralite (diethyldiphenylurea), and 2,4-dinitrotoluene were biotransformed. These results suggest that these compounds may be successfully removed in a continuous treatment process operated in both aerobic and anaerobic modes. High nitrate concentrations are reduced to acceptable levels. All compounds studied were attacked under one set of conditions or another. The fate of several of the transformation products is not known because of an administrative decision to terminate the work on this project before completion. Although increasing the concentrations of nutrients or carbon source supplements increased the disappearance of a compound it is hoped that suitable adjustments in operating parameters of a continuous system would make it possible to drive the disappearance to completion. Some intermediates were detectable only after a several thousand fold concentration. This would place their residual concentration in the ppb range in our idealized laboratory system, which may satisfy regulatory requirements for some of the compounds.

This document reports research undertaken at the US Army Natick Research and Development Command and has been assigned No. NATICK/TR-85/050 in the series of reports approved for publication.

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